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TRACHOMA IN EGYPT

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Trachoma as it occurs in Egypt is true trachoma and is identical with the disease existing in America among the Indian and white races.[1] The minor differences which are exhibited by individual patients in Egypt or in America are based, in part, on the greater chronicity of the disease in the Orient and the more active type of infection in the West. This view is supported by the observations of ophthalmologists in Egypt, who declare that while natives appear to tolerate trachoma with a definite degree of resistance to its harmful effects, Occidentals infected in the Orient show no such defense. In the latter, the disease presents a more acute and extensive inflammatory character.

On the other hand, certain clinicians regard "trachoma" as an inclusive term, or, as expressed by Wilson:[1a]

> A definition though now applicable only to trachoma may in the future be found to describe only a syndrome which may be common to several aetiologically distinct conditions or to the complications of a simpler condition.

However this may be, we have observed cases in Cairo which correspond in all respects to those seen in New York. The latter in turn

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The officers of the Department of Public Health in the service of His Majesty, King Fuad I, His Excellency, Dr. Mohammed Shahin Pasha, Under Secretary of State, and the Members of His Excellency's Staff offered every facility for conducting this study. At the Memorial Ophthalmic Laboratory we enjoyed the privilege of working in one of the best equipped ophthalmic institutions, a modern and progressive laboratory, under the direction of Dr. Rowland P. Wilson. Dr. Wilson and his Staff, Major F. H. Stewart, Dr. A. F. Abassi and Dr. F. el-Tobgy, welcomed us cordially and gave whole-hearted assistance. Dr. H. M. Dewairy supplied us with some of our clinical material.

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1. (*a*) Wilson, R. P.: Sixth Annual Report of the Giza Memorial Ophthalmic Laboratory, Cairo, Egypt, Schindler's Press, 1932. (*b*) Thygeson, P.: Am. J. Ophth. **14:** 1104, 1931.

exhibit all the clinical variations found in the four main types occurring in Egypt as they were originally described by MacCallan.[2]

Bacteriologic studies were undertaken to determine the flora of local trachomatous lesions and to compare it with that which has been found in New York. Investigations were also made with the view of disclosing, if possible, any particular agent which might have a causal relation to the Egyptian disease.

The tarsectomized tissue obtained in Cairo from uncomplicated trachoma yielded, as a rule, fewer varieties and less profuse growth of bacteria than were found in similar New York material. In Egypt, xerosis, Morax-Axenfeld and Koch-Weeks bacilli, pneumococci, streptococci (usually of the indifferent types) and gonoccoci were observed in stained film preparations or in cultures more frequently than in New York. In New York, on the contrary, the numerous varieties of chromogenic, gram-negative bacilli, diphtheroids and staphylococci were encountered.

A search for a hitherto undescribed microbic agent in trachoma as it exists in Egypt led us to no result worthy of description. This investigation was based on the examination of film preparations derived from uncomplicated and complicated lesions of the different stages of the disease, forty-six specimens in all. In addition, the tissue sections stained with hematoxylin and eosin and the Gram and Giemsa stains from twenty such cases were studied; and the conjunctival tissue or grattage material obtained from twenty-four patients was cultivated in leptospira medium and on horse-blood agar, by methods to be described.

We have discussed in a previous article[3] the problem of a filtrable or ultramicroscopic virus as the incitant of trachoma, and have shown from our experiments and from those of others that such a virus has not as yet been detected. Moreover, Stewart, of the Giza laboratory, will soon report negative filtration experiments with trachomatous material of Egyptian origin.

2. (*a*) Attention should be directed to the fact that almost all cases of trachoma observed by us in New York occurred among Europeans, and that in most of the patients the disease could have arisen before their emigration. (*b*) MacCallan, A. F.: Lancet **2:** 919 (Oct. 30) 1926; Trachoma and Its Complications in Egypt, London, Cambridge University Press, 1913, p. 1.

3. Olitsky, P. K.; Knutti, R. E., and Tyler, J. R.: J. Exper. Med. **54:** 557, 1931.

Inclusion Bodies in Egyptian Trachoma

We have stated[3] that inclusion bodies of the kind characteristic of many ultramicroscopic viruses were not found in our examinations of New York cases of trachoma. Nor have we met with the so-called inclusion bodies of Prowazek-Halberstaedter (known as Prowazek bodies). The probability of these structures being of bacterial origin and not specific for trachoma was also discussed.[4]

Stewart[5] found that definite Prowazek inclusions are absent, as a rule, in the uncomplicated type of trachoma prevailing in Egypt. On the other hand, he concluded that they are present in most cases complicated by secondary infections, especially those induced by such pathogenic bacteria as Koch-Weeks bacilli, Morax-Axenfeld bacilli, pneumococci, streptococci and gonococci. Our studies, although based on a much smaller number of observations, tend to support Stewart's findings. For example, in film preparations derived from twenty-two patients having uncomplicated trachoma (mostly of MacCallan's types I and IIa), no single case revealed Prowazek bodies; yet in similar examinations of specimens from twenty-one cases of trachoma showing superinfection by Koch-Weeks bacilli, fourteen showed definite bodies. In addition, two of four patients having trachoma with secondary Morax-Axenfeld bacilli and one with hemolytic streptococcic infections exhibited the inclusion corpuscles. The bodies were not, however, found in stained tissue sections derived from either complicated or uncomplicated trachomatous conjunctivitis. In all of these observations a distinction was made between characteristic Prowazek structures and other inclusions, particularly intracellular bacteria of ordinary species.

Wilson,[1a] in summing up the results of an analysis of examinations of trachomatous tissues and secretions for Prowazek bodies, stated:

> In Egypt the home of trachoma they are but rarely found. We therefore cannot understand how some have found them in 100 per cent of cases. We cannot believe, as it is said, that it is merely a question of the time spent over each individ-

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4. Olitsky, P. K.: Rev. internat. du trachome **7**: 173, 1930. Compare particularly Bengtson, I. A.: Am. J. Ophth. **12**: 637, 1929, and Science **77**: 218, 1933; also, Williams, A. W.: J. Infect. Dis. **14**: 261, 1914. Olitsky, Knutti and Tyler.[3]

5. Stewart, F. H., cited by Wilson [1a]. A report elaborating these earlier results and based on several hundred additional observations is to be published.

ual case but rather we believe that there is not sufficient discrimination of the various epithelial inclusions and pseudo-inclusions which are so commonly met with.

From our experience in the Orient, we can confirm the opinion of Wilson and his associates[6] and of others[4] concerning the essential bacterial structure of the elements composing the characteristic Prowazek bodies, and conclude with Stewart that their presence is correlated with secondary bacterial infection superimposed on trachomatous lesions.

It appears, then, from studies carried out in Egypt by others and ourselves that trachoma, as it occurs there, is similar to the disease in New York, and that no fact could be brought to light revealing the identity of a hitherto unrecognized microbic or ultramicroscopic incitant.

During the course of the cultivation experiments, we isolated Bacterium granulosis. Since that micro-organism had not previously been found in Egypt[1] and in view of the significance attached to it in its relation to human trachoma existing elsewhere,[7] the finding takes on greater interest.

Recovery of Bacterium Granulosis

Methods.—We have followed in the cultivation experiments the mode of procedure originally devised by Noguchi[7a] in his studies on trachoma. We have employed particularly the two mediums of Noguchi, the so-called leptospira medium and horse-blood agar, as modified by Olitsky, Syverton and Tyler.[8] The tissue itself and decimal dilutions of the suspension of ground conjunctival material up to 1:1,000 were used for inoculation. The average number of tubes of leptospira medium and blood-agar plates which served for primary inoculations in each case was eight tubes and ten plates. Following the usual practice, we examined at intervals each inoculated leptospira tube by means of (*a*) the dark-field microscope, (*b*) transplants to blood-agar plates and (*c*) a Gram stain of its contents.

6. Personal communication to the authors.

7. (*a*) Noguchi, Hideyo: J. Exper. Med. (supp. 2) **48:** 1, 1928. (*b*) Olitsky, P. K.: Tr. Am. Acad. Ophth. **35:** 225, 1930.

8. Olitsky, P. K.; Syverton, J. T., and Tyler, J. R.: J. Exper. Med. **57:** 871, 1933.

Contamination of Cultures by "Nile Dust."—Contamination of cultures by dust or sand is generally encountered in countries having the physical geography of Egypt. In two preliminary cultivation tests made in an open room without vigilant control of the atmosphere, we found, especially on agar plates, such marked contamination with molds as to render cultures so made useless for study. We attempted, in the absence of a dust-proof cubicle or chamber, to control the dust in the air by keeping the windows and the door shut, spraying the room with an oily vapor and wetting the work table with a 1:1,000 solution of corrosive mercuric chloride. In addition, a glass box was provided, the inner surfaces of which were frequently washed with the solution, and which admitted only the worker's hands and forearms. In this small chamber most of the mediums were tubed or plated, and all of the inoculations were made except those in the leptospira medium. In the end this method served to give no more contamination than is ordinarily met with in our cultural work in New York.

Materials.—In a certain number of patients with trachoma tarsectomy is the therapeutic indication. The operation is performed as follows: After local anesthesia produced with procaine hydrochloride, the conjunctival sac is thoroughly washed by means of warm, sterile physiologic solution of sodium chloride, and the excision is made under strict aseptic conditions.

Only the removed tarsal plate with its overlying mucosa comprised the material used for cultivation tests. In addition to the two preliminary cases mentioned in the preceding paragraphs, we obtained thirteen such tarsi. These were derived from the same number of patients having trachoma chiefly of types I, IIa and IIb of MacCallan's designations.[2b] Of the thirteen, however, two tarsi offered control material, for they were removed from patients having type III trachoma;[9] that is, they showed follicles in the retrotarsal fold but not over the diffusely scarred tarsal plates.

9. Briefly, these types of MacCallan, so widely used now by clinicians in the diagnosis of trachoma, describe: the early disease with its initial follicles, which in Egypt first appear over the tarsus, and in the Occident, in the fornix (type I); the intermediate stage, in which follicles predominate (type IIa) or papillary hypertrophy develops along with the follicles (type IIb), and a later stage, of beginning cicatrization (type III).

Use of Tarsectomized Tissue.—Noguchi and ourselves have employed for cultivation and transmission experiments tarsectomized tissue, since it is more suitable for these purposes. Also, Bengtson[10] has recently stated that the chances for successful transmission to animals and for isolation of Bacterium granulosis are greatly enhanced by the use of the larger amounts of material yielded by excised tarsi. It is noteworthy that prior tests in Egypt by others[1] employing chiefly material expressed from follicles or procured by means of conjunctival grattage failed to yield positive cultures.

Besides the studies made with excised tissue, we cultivated the substance obtained by conjunctival grattage—an operation performed for therapeutic purposes—from four added cases of types I and IIa trachoma. Contamination by many different kinds of micro-organisms, especially molds, was so frequent and showed itself by overgrowth of cultures so early, within from twenty-four to forty-eight hours, that these cultures were found valueless for the isolation of fragile and slowly growing bacteria. In any event, Bacterium granulosis was not recovered from the scraped conjunctival material. In performing cultivation tests, particularly in Egypt, it is therefore essential to pay special attention to the bacteriologic cleanliness of the technic, and, *pari passu*, to use tarsectomized tissue as the inoculum.

Identification of Cultures.—The morphology of Bacterium granulosis and its cultural characteristics in the leptospira medium, on horse-blood agar and on plain agar are distinctive; these were the criteria for its identification. Fermentation reactions with different carbohydrates were not studied in view of the irregular results which were previously obtained in different tests with the same strains of bacteria. The final determination of the species of micro-organism involved was made by means of serologic examination. We used for this purpose specific rabbit agglutinating antiserum of a titer of 1:10,000 prepared with a strain of Bacterium granulosis isolated from a case of trachoma in New York. In all instances in which a culture was finally designated as that of Bacterium granulosis, the serum agglutinated such a strain in a dilution of at least 1:1,000. Indeed, no morphologic, cultural or serologic difference could be shown to exist between the Giza strains of granulosis organism and other cultures now in our possession. The latter cul-

10. Bengtson, I. A.: Science **77**: 218, 1933.

tures were ultimately recovered from patients having trachoma who resided in America (New York, Chicago, St. Louis, Denver and Albuquerque, Fort Defiance and Leupp, Ariz.), in Tunis and in Italy. The Egyptian strains were also found to be identical with those isolated in New York from the conjunctival tissue of monkeys having granular conjunctivitis as a result of inoculation of human trachomatous tissues or cultures of Bacterium granulosis.

It should be emphasized that only from one to four colonies of Bacterium granulosis could be found on an initial plate culture. In other cases, the growth was sometimes seen as an offshoot from a larger colony of another micro-organism, usually a staphylococcus, in which case a quarter moon-shaped excrescence arose from the margin of the larger colony. Under such conditions, Bacterium granulosis could easily be overlooked. In still other instances, overgrowth at the site of a colony of Bacterium granulosis rapidly took place from adjacent micro-organisms of other species, when complete suppression or disappearance of the granulosis organism occurred. Thus, because of sparse growth, symbiosis with other colonies and overgrowth or suppression of the slowly growing Bacterium granulosis by more rapidly growing bacteria or molds, sources of error may arise and account for discrepancies in the efforts at isolation by different experimenters.

Numerical Results of Cultures.[11] The tarsectomized tissue for cultivation tests was procured from two patients with trachoma having scarred conjunctivae and from eleven showing a preponderance of follicles.

The two scarred conjunctival tissues yielded only a few microorganisms both in the leptospira medium and on blood-agar plates. These were mainly molds and diphtheroids and probably arose from sources other than the tissues themselves, for in the sections stained

11. In addition to the number of tarsectomized tissues cultivated as mentioned, there were five which were received in the last days of our stay in Giza. Three of them were rapidly overgrown by molds, owing to contamination of the tissue itself before cultivation, and two could not be studied thoroughly owing to lack of time. In none of the five was any organism noted during the period of observation which deserved special attention. In ordinary routine practice, a culture is usually studied for an average period of twelve days from the time of initial inoculation, unless an earlier positive result is obtained.

by the Gram and Giemsa stains, no organisms could be found. Bacterium granulosis was not recovered in these two instances.

Of the eleven tarsi removed from patients with trachoma showing the follicular type I or IIa disease, four yielded cultures of Bacterium granulosis.

The first positive result was observed in the case of an Egyptian woman, aged 25, who had an attack of acute follicular trachoma (type I) for a month prior to tarsectomy. The acute disease was superimposed on a preexisting type IIa trachoma and was characterized by pannus tenuis of indefinite duration but assumed from the patient's history to have existed for several years. Bacterium granulosis was recovered from the blood-agar plate culture. A morphologically similar organism also appeared in the leptospira medium, but this was not followed after a pure culture was obtained from the plate. The associated organisms were staphylococci, diphtheroids and gram-negative diplobacilli.

The second positive culture was obtained from an excised tarsus of an Egyptian girl, aged 13 years. She had trachoma, probably since infancy, and at the time of tarsectomy the disease was manifest as type IIa with an added secondary infection caused by Koch-Weeks bacilli. The patient exhibited injected palpebral and bulbar conjunctivae, a mucopurulent secretion, phlyctenules at the limbus and in the palpebral conjunctivae, follicles and fine, streaked scars. The condition was accompanied by pannus crassus. The leptospira medium and plate cultures yielded Bacterium granulosis and only one associated organism, the Koch-Weeks bacillus.

The third culture of the granulosis organism occurred in the case of an Egyptian boy, aged 8 years, who had trachoma (type I) associated with secretion, Herbert's pits and pannus tenuis. The disease may have endured since infancy. The micro-organism was recovered only from the plate cultures; the concomitant bacteria met with were Bacillus xerosis and staphylococci.

The fourth and last positive culture was obtained in the case of an Egyptian girl, aged 12 years, who had trachoma probably since infancy and exhibited definitely the type IIa disease. Follicles predominated in the lesions, and Herbert's pits and pannus tenuis were also visible. Bacterium granulosis was isolated on the blood-agar plates five days after seeding; the inoculated leptospira medium was not studied. The associated micro-organisms in this case were Morax-Axenfeld bacilli and molds.

In sections of the conjunctival tissues secured from the aforementioned eleven cases of trachoma and stained by the Gram and Giemsa stains, we found w thin the lesions small, gram-negative bacilli lying singly or in clumps of from 3 to 6 organisms. Morphologically, they resembled Bacterium granulosis and were observed in the four tissues from which cultures of Bacterium were obtained as well as in four in which such cultures failed.

Inoculation of Monkeys.—During our stay in Egypt, there were available for our use only four Macacus sinicus monkeys.

The monkeys had smooth conjunctivae, and each was inoculated in the upper left lid, after complete ether anesthesia, with two pooled Giza strains (nos. 5 and 6) of Bacterium granulosis. Two-tenths cubic centimeter of the bacterial suspension was injected subconjunctivally, followed by scarification in accordance with the usual procedure.[7a]

Monkey A: After seven days this animal exhibited in the upper part of the left conjunctiva several small, discrete follicles accompanied by redness, congestion, edema and roughness of the membrane. These lesions became more marked, particularly with regard to enlargement of the follicles, during the course of observation extending over the following two weeks. Fourteen days after the first appearance of the reaction in the inoculated conjunctiva, the right, or uninoculated, lid showed several small and large follicles, redness, roughness, edema and congestion.

Monkey B: This monkey reacted similarly to monkey A, with the exception that a mucopurulent secretion accompanied the lesions in the two eyes.

Monkey C: Two weeks after inoculation granular conjunctivitis developed in both the inoculated and the uninoculated conjunctiva, but the signs were more marked in the former. After another week, secretion accompanied the lesions in both eyes, and definite progression and extension of the conjunctival changes were noted.

Monkey D: The first changes of granular conjunctivitis in the inoculated conjunctiva appeared two weeks after inoculation, and extension to the uninoculated eye occurred a week later. In this animal a mucopurulent secretion was also observed.

It appears, then, that the Giza strains of Bacterium granulosis induce in the sinicus monkeys a progressive granular conjunctivitis, first appearing, from seven to fourteen days after injection, in the inoculated lid, and then spreading by contact, within a period up to two weeks later, to the uninoculated eye. The general appearance of the disease produced experimentally in Macacus sinicus monkeys is similar, though less marked, to that produced in New York in rhesus monkeys by other occidental strains of Bacterium granulosis.[12]

12. On our return to New York, we pooled the four Egyptian strains and inoculated subconjunctivally with this material five Macacus rhesus monkeys. Within from six to eighteen days four of the animals had characteristic progressive granular conjunctivitis.

SUMMARY AND CONCLUSIONS

The bacteriologic studies of trachoma occurring in Egypt led us to conclude that no essential differences exist between the disease encountered there and the disease existing in the United States. The minor differences may be ascribed to a variation of the terrain or to racial susceptibility. The disease is more prevalent in Egypt, and the native adapts himself to its ravages with a greater degree of resistance.

In our studies made in New York we have failed, with the exception of confirming Noguchi's conclusions concerning Bacterium granulosis, to implicate another factor in a causal relation to the disease. So also in our studies made in Egypt, while more light was cast on the bacteriologic problem of the disease, they did not disclose evidence which might change the status already established for Bacterium granulosis in trachoma in human beings.

That Bacterium granulosis can be isolated from certain cases of trachoma as it occurs in Egypt by the methods we have herewith described is now apparent. The organisms thus recovered are evidently identical in morphologic, cultural and serologic characteristics with those obtained from patients having trachoma and residing in other parts of the world. The Egyptian strains of Bacterium granulosis are also pathogenic for monkeys in practically the same way as are the cultures derived from occidental sources.

In view of our failure to discover in Egypt any other causal agent of the disease, and from the positive findings regarding Bacterium granulosis, the original conclusions of Noguchi on the causal relation of Bacterium granulosis to trachoma in human beings have received additional support.

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FURTHER STUDIES ON THE HYPOPHYSEAL SUBSTANCE GIVING INCREASED GONADOTROPIC EFFECTS WHEN COMBINED WITH PROLAN*

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(Received for publication, June 28, 1933)

In a previous study (1) of the greatly increased gonadotropic effect of prolan when it is combined with some component of the anterior hypophysis, it was pointed out that the hypophyseal substance in question could be neither the growth nor gonad-stimulating hormone. Indeed, at that time it seemed that this substance had no physiological effect of its own, but was dependent upon its combination with prolan for the exhibition of activity. It can now be shown that the substance (the so called synergic factor) exhibits peculiar gonadotropic effects which distinguish it from any substance hitherto isolated from the hypophysis. When it is administered alone (*i.e.* uncombined with prolan) in three daily injections with autopsy of the animals at the end of 96 hours, it has produced little or no enlargement of the ovaries; yet sacrifice of the experimental animals at 36, 60 and 84 hours (Chart 1) shows that definite gonadotropic properties are possessed by this material, evidenced by slightly increased ovary weights as early as the 36th hour after the beginning of dosage, an effect earlier than that secured by some of the most potent gonadotropic preparations known. For example, the remarkable gonad-stimulating hormone from pregnant mare's serum produces a slower initial development of the ovary,

* Aided by grants from the Rockefeller Foundation and from the Committee for Research in Problems of Sex of the National Research Council. We desire here also to express our thanks to the I. G. of Elberfeld, Germany, who, through Drs. H. Hörlein, W. Schulemann and F. Laqueur, placed generous amounts of prolan at our disposal, and finally, to Eli Lilly and Company of Indianapolis, without whose assistance in securing hypophyseal products this research would have been impossible.

which increases rapidly after about 72 hours so that the maximum effect from three daily doses is produced in 96–120 hours. Prolan is similar in its insignificant early effect on the ovary but the ovary weights plateau after 72 hours. These types of ovarian development are shown graphically in Chart 2 (*a*) and the comparison of prolan,

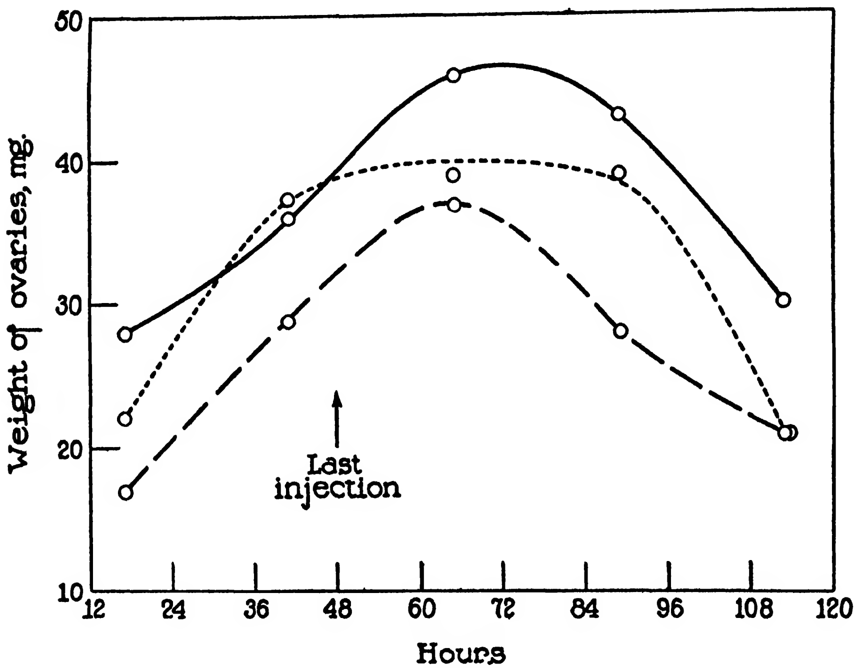

CHART 1. Early response of the ovary to the synergistic factor followed by regression within 96–120 hours. The preparations indicated have been subjected to different treatments:

——— Glacial acetic acid (R3521).

-------- Isoelectric precipitation (supernatant) (R3523).

— — — Trypsin and erepsin digestion (R3522).

synergic factor, synergic factor in combination with prolan, and pregnant mare's serum may be made.

When the injections are continued for a total of 6 days (instead of the usual three day injections with autopsy at 96 hours) the development produced in the ovaries by the synergic factor does not regress but is maintained. Chart 2 (*b*) shows the ovarian weights produced

by this method of injection using the same gonad-stimulating substances as before. In the cases where the synergic substance was injected for 6 days (twice daily, as in Chart 2 (*a*)), the ovary weights do not decrease after 72–84 hours as in the case of the three injections.

The increased ovary weights are at first not paralleled by clear morphological expression. Even though increase in ovarian weight is

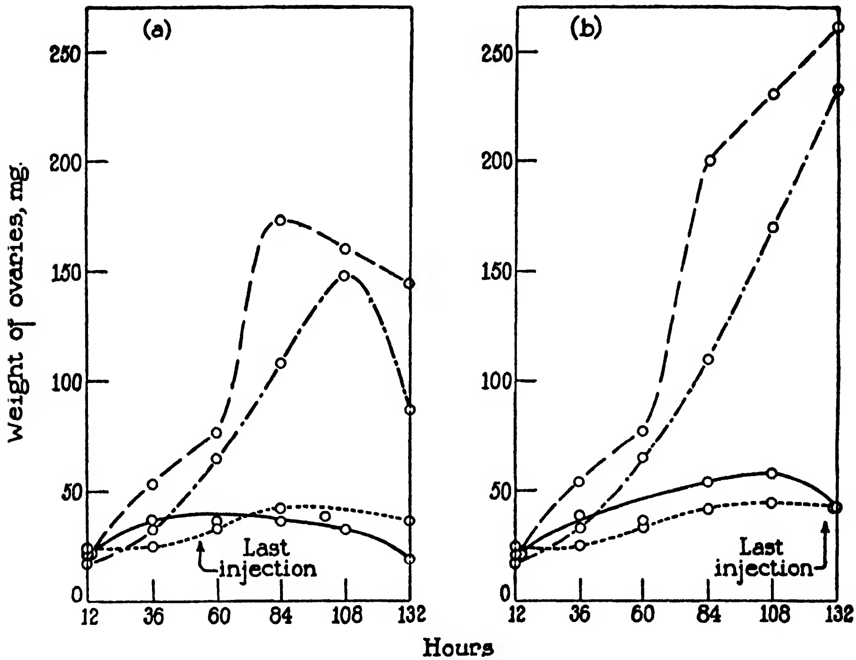

CHART 2. Response of the ovary to various gonadotropic hormones with (*a*) injection for 3 days (twice daily) and (*b*) injection continued for 6 days.

——— Synergic factor (R2721).
- - - - - - - - Prolan.
— — — Synergic factor in combination with prolan.
—·—·— Pregnant mare serum.

noted before 36 hours, the ovary may show only a slightly increased vascularity. An increase in the number of medium sized follicles is, however, usually noted by 24–48 hours. Some ovaries show no further development even by 113 hours. This is true for example of the synergic fraction prepared by trypsin and erepsin digestion

(R3522) shown in Chart 1. However, the ovaries of animals injected with the synergic factor more frequently show by 40–84 hours at least a few intermediate bodies or small and medium corpora. Ovulation has never been noted. Uterine development, vascularization and increase in size, is often observed by 24–48 hours.

Non-Identity of the Synergic Factor with Other Hypophyseal Substances

In 1931 Fevold, Hisaw and Leonard of Wisconsin (2) reported the fractionation of pyridine extracts of the entire hypophysis into follicle-stimulating and luteinizing components, the latter fraction without detectable action on the ovary when administered alone; but when combined with the former, it increased markedly its action. Indeed, the whole efficacy of unfractionated pyridine extracts was again secured by recombination of the two fractions. The recombination phenomenon resembles so strikingly what we have described as the activation phenomenon resulting from the addition of prolan to some hypophyseal substance, that an examination of the two procedures for possible underlying identity is important. Furthermore, Leonard (3) has shown that the follicle-stimulating fraction of Fevold, Hisaw and Leonard (2) gave augmentation when combined with prolan. Hisaw and his collaborators have also pointed out (4) that prolan and their luteinizing fraction behave similarly in augmenting the action of the follicle-stimulating fraction. In comparing the properties of the hypophyseal synergic factor prepared in this laboratory with the properties ascribed to the follicle-stimulating fraction (2, 5) several differences became apparent. Firstly, the synergic factor given in increasing doses, though producing definite ovarian development in 96 hours, does not give the large ovaries described for the follicle-stimulating fraction. In Table I, it is seen that a dose of the synergic principle 100 times that necessary to give activation (the minimal effective dose) produces only moderate ovarian development.

Secondly, the synergic factor which is very potent in its ability to augment the action of prolan gives only moderate ovary weights (Table II) when combined with the luteinizing fraction. However, the follicle-stimulating fraction is augmented by both the luteinizing fraction and prolan (4), producing large ovary weight as well as high percentage activation.

Most fractions of the synergic factor so far obtained give some luteinization of the ovaries in 96 hours although uniform enlargement of the follicles to medium sized structures is the chief effect. Whether

TABLE I

Gonad-Stimulating Action of Large Doses of the Synergic Factor

Synergic factor			Prolan		Combination (*in vitro*)	
Preparation	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
Aqueous alcohol (pig)	0.27	18			54	25
R2586	0.54	20	13.6	43	102	126
	1.36	25			129	158
Aqueous alcohol (pig)	1.36	22			107	181
R3524	2.73	28			130	195
	6.8	27	13.6	34	128	197
	13.6	26			118	181
	27.3	36			131	152
	54.5	67			141	72

TABLE II

Combination of Luteinizing Fraction with the Synergic Factor

Synergic factor			Luteinizing fraction		Combination (*in vitro*)	
Preparation	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
Aqueous alcohol (pig) R2834,	2.7	26	13.6	22	41	36
R3160	1.36	21	27.3	23	94	261
Aqueous alcohol (sheep), supernatant after isoelectric precipitation R3330	0.27	17	27.3	23	64	178
Alkaline extract (sheep), trypsin and erepsin digested R3331	1.36	25	27.3	23	80	166
Acetic acid powder (sheep) R3332	13.6	19	27.3	23	54	125

the luteinization is an inherent property of the substance, is due to contamination by the luteinizing fraction or is due to the animal's own hypophysis cannot be said at present.

These facts clearly indicate that the synergic factor, prepared in this laboratory, cannot be identified with either the luteinizing or follicle-stimulating fractions and is actually a third gonadotropic substance from the pituitary. *Indeed, it would appear that the follicle-stimulating fraction (2, 5) is a mixture of the synergic factor herein described, with what has been designated as the follicle-stimulating substance.* There is no question as to the non-identity of prolan and the luteinizing fraction.

It would appear, then, that we are not justified in identifying the above mentioned recombination effects with our activation experiments, that indeed a comparison of the two experiences cannot as yet be instituted, although future research may disclose an underlying reason why the combinations in question should necessarily increase in each instance the gonadotropic effect.

Antagonism of Gonad-Stimulating Effects by Some Hypophyseal Component

Some years ago it was shown that the normal gonad-stimulating effect of implants of anterior hypophysis in hypophysectomized rats (Smith (6)) or normal immature rats (Evans and Simpson (7)) could be prevented by the simultaneous intraperitoneal injection of crude extracts of anterior hypophysis. Leonard (8) has shown similarly that simultaneous injection of Van Dyke's growth extract effectively masked the action of the follicle-stimulating fraction.

It may be noted here that the conditions employed for obtaining synergism are by subcutaneous injection of the *in vitro* combination of prolan with the synergic factor. The conditions used for demonstration of the antagonism phenomenon were subcutaneous injection of prolan and simultaneous intraperitoneal injection of the hypophyseal component.

In this investigation, the capacity of fractions containing the synergic factor to decrease the action of potent gonad-stimulators was determined and this property was almost invariably a concomitant of the synergistic activity (Table III). However, when digestion of anterior lobe was carried out (digestion with trypsin followed by a short digestion with erepsin) the two activities were differentially separated. The synergic factor was not injured but the digested ma-

terial was no longer able to influence adversely the gonadotropic effect of prolan. Similarly an isoelectric precipitation at pH 4.4–4.6 of

TABLE III

Antagonism and Synergism of Prolan and Anterior Hypophyseal Fractions with Subsequent Differential Destruction of the Antagonistic Factor

Preparation	Antagonism phenomenon					Synergism phenomenon					
	Hypophyseal fraction (intraperitoneal)		Prolan (subcutaneous)		Combination (for antagonism)	Hypophyseal fraction (subcutaneous)		Prolan (subcutaneous)		Combination (for synergism)	
	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg*	*per cent*
Aqueous alcohol (pig); control, R3116	2.7	17	13.6	43	16	2.7	25	13.6	43	124	**148**
As above, pH 4.5, 70°C., 1 hr.					27					124	**148**
" " pH 4.5, 70° " 3 hrs.					32					93	**86**
" " pH 8.5, 70° " 1 hr.					27					79	**58**
" " pH 8.5, 70° " 3 hrs.					35					54	**8**
Aqueous alcohol (sheep); control, R2289	2.7	25	13.6	43	24	1.36	21	13.6	38	137	**234**
As above, supernatant after isoelectric precipitation R3143	1.36	20			60	0.54		13.6	43	175	**280**
Trypsin and erepsin digest (sheep) R3356	1.36	19	13.6	45	46	1.36	25	13.6	31	144	**278**
	cc.										
Alkaline extract (sheep); control, R2889	1.5	9	13.6	30	20	2.7	63	13.6	35	178	**122**
						0.27	23			105	**162**
Above solution, trypsin digested	1.5	9			24	2.7	33			143	**186**
						0.27	20			64	**73**
Trypsin digestion followed by erepsin	1.5	12			47	2.7	37			153	**183**
						0.27	22			59	**51**

fractions exhibiting both phenomena left a supernatant liquor which was the most potent fraction in synergistic activity (Table VII) yet obtained, but was unable to decrease the effect of prolan. These

experiments (summarized in Table III) strongly indicate that the two properties are not due to the same constituent.

The fact that these fractions contain little or no growth hormone and do not contain the luteinizing or follicle-stimulating fractions would seem to eliminate the synergic material and these other substances from consideration as the antagonist.

TABLE IV

Combination of Synergic Factor with Prolan Prepared by Different Methods

Synergic factor			Prolan			Combination (*in vitro*)	
Preparation	Dose	Weight of ovaries	Preparation	Dose	Weight of ovaries	Weight of ovaries	Activation
	mg.	*mg.*		*mg.*	*mg.*	*mg.*	*per cent*
Aqueous alcohol (sheep)	6.8	29	Alcohol-ammonia R2193	27.3	47	221	**281**
			Repeated alcohol precipitation R2194	13.6	31	111	**164**
				27.3	42	165	**211**
Aqueous alcohol (pig)	2.7	17	Alcohol-ammonia R2380	27.3	35	155	**343**
			Repeated alcohol precipitation R2381	54.5	33	136	**312**

Optimal Conditions for the Demonstration of Synergism

It seemed of interest to determine the effect of prolan variously prepared on the degree of synergism obtained. In this respect, prolan prepared by the alcohol-ammonia extraction method (9) was found to be much more potent than that prepared by repeated alcohol precipitation (see Table IV).

In order to determine the optimal proportions of prolan and the synergic factor, a constant amount of prolan was used in combina-

tion with varying amounts of the synergic principle and a constant amount of the synergic principle was used in combination with varying amounts of prolan. The results are given in Tables I and V. It is seen that for optimal augmentation between 10 and 20 times the

TABLE V

Combination of Variable Amounts of Prolan with Constant Amounts of Synergic Factor

Synergic factor			Prolan			Combination (*in vitro*)	
Preparation	Dose	Weight of ovaries	Dose	Weight of ovaries	Description of ovaries	Weight of ovaries	Activation
	mg.	*mg*	*mg.*	*mg.*		*mg.*	*per cent*
Aqueous alcohol (pig) R2739	1.36	22	0.54	26	Corpora, large follicles	33	**10**
			2.7	26	" "	108	**260**
			6.8	36	" "	114	**185**
			13.6	38	" "	130	**210**
			27.3	38	" "	187	**345**
			81 8	49	" "	209	**294**
	2.73	25	0.54	26	" "	44	**33**
			2.7	26	" "	91	**176**
			6.8	36	" "	154	**258**
			13.6	38	" "	131	**191**
			27.3	38	" "	158	**251**
			81.8	49	" "	105	**87**
Aqueous alcohol (pig) R3082	2.73	21	0.27	15	Infantile	26	**24**
			0.54	29	Corpora	37	**16**
			1.36	32	"	60	**71**
			2.73	30	"	75	**127**
			6.8	44	Corpora, large follicles	128	**172**
			13.6	43	" "	155	**237**

minimal dose of prolan (gonadotropic assay) is necessary in combination with about two to three times the minimum dose of the synergic factor (activation assay). The use of larger doses of the synergic factor is inadvisable, since the augmentation produced is not as great

as that given by the lower doses. This may possibly be due to the presence of the antagonistic substance (*vide supra*) as a contaminant in the preparations then employed.

As to duration of the synergic experiments, Chart 2 indicates that a sufficient augmentation (with three injections) is obtained in 72 hours which is, therefore, not too early to terminate the experiment.

Chemical Characteristics of the Synergic Factor

The comparative stability of the synergic substance noted in the previous paper (1) has been shown to extend over a wide variety of hydrogen ion concentrations, even at slightly elevated temperatures. Thus in alkaline solution pH 8–12 it was stable at room temperature for 24 hours. At pH 8.5 it was seriously injured in 1–3 hours at 70°C. but at 37°C. it was stable for 6 hours. In acid solutions (pH 4–1) it was somewhat destroyed in 24 hours at room temperature. At pH 4–5 it was quite stable for 1–5 hours at 37°C., but 9 hours at pH 2 completely inactivated the material. Stability to acid and alkali is illustrated in Tables III and VI.

Enzymatic hydrolysis of anterior lobe material was next undertaken in order to remove as far as possible protein contaminants without destruction of the synergic principle. Pepsin was found to be unsatisfactory since the synergic factor was readily injured. Trypsin, however, was found to be quite advantageous since 4–5 hours digestion at 37°C. hydrolyzed a considerable portion of the protein, but did not affect the activity of the synergic principle. When the tryptic digestion was followed by erepsin, though some further hydrolysis was effected, there was little loss in activity. The erepsin digestion was of value since the antagonistic factor was thereby eliminated. The action of various digests is shown in Table VI.

Following tryptic and ereptic digestion, the solution could be concentrated *in vacuo* to one-tenth the original volume and then the addition of alcohol to a concentration of 80 per cent precipitated the active material which was potent for synergism in 0.27 mg. dose (3700 rat units per gm.). This material gave a positive biuret test.

The most convenient method of preparing a potent fraction of the synergic principle is the aqueous alcohol extraction procedure previ-

ously described (1). The material, however, contained appreciable amounts of the antagonistic factor among other possible contaminants.

Isoelectric precipitation of impurities was efficacious both in the elimination of the antagonistic factor and in producing the most potent

TABLE VI

Digestion of Sheep Anterior Pituitary Powder with Trypsin, Erepsin and Pepsin

Hypophyseal material			Prolan		Combination (*in vitro*)	
Preparation	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
	cc.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
Alkaline extract, neutralized, frozen (control) R2798	2.7	63	13.6	32	178	**131**
	0.27	23			105	**183**
Alkaline extract (above), pH 8.5, 37°C. for 4.5 hrs. with trypsin R2803	2.7	33	13 6	32	143	**204**
	0.27	20			64	**88**
Trypsin digest (above), pH 8.0, 37°C. for 4 hrs. with erepsin R2807	2.7	37	13.6	32	153	**200**
	0.27	22			59	**64**
Alkaline extract, pH 4.0, 37°C. for 4.5 hrs. (control) R2811	2.7	23	13.6	32	139	**276**
	0.27	18			49	**53**
Alkaline extract, pH 4.0, 37°C. for 4.5 hrs. with pepsin R2813	2.7	18	13.6	32	46	**43**
	0.27	19			41	**24**
Aqueous alcohol extract water solution, neutral, frozen (control) R2711	0.27	32	13.6	38	140	**169**
Aqueous alcohol extract, water solution, pH 8.5, 37°C. for 6 hrs. (control) R2712	0.27	48	13.6	38	140	**106**
Aqueous alcohol extract, water solution, pH 8.5, 37°C. for 6 hours with trypsin R2713	0.27	28	13.6	38	196	**308**
Trypsin digest (above), pH 8.0, 3 hrs. with erepsin R2717	0.27	26	13.6	38	140	**205**

fraction yet obtained. A potent fraction of the synergic factor (containing 7300 rat units per gm.) was precipitated isoelectrically and the supernatant liquor contained the active material which was precipitable

by alcohol. This material was potent in a total dose of 27 *gamma* (37,000 rat units per gm.). It still gave a biuret test. The biological assay of the active fractions described above (from digestion and from isoelectric precipitation) is shown in Table VII.

TABLE VII

Assay of Potent Fractions of the Synergistic Principle

Synergic principle			Prolan		Combination (*in vitro*)	
Preparation	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
Concentrate after tryptic and	1.36	25			144	**278**
ereptic digestion (sheep)	0.54	—	13.6	31	134	**252**
R3023	0.27	—			73	**92**
	0.136	—			46	**21**
Aqueous alcohol (sheep)	0.27	28			125	**127**
R3372	0.136	21	13.6	45	114	**137**
	0.54	19			57	**24**
Isoelectric supernatant from	0.011	32			143	**198**
aqueous alcohol extract	0.082	25	13.6	34	109	**180**
(sheep) R3500	0.054	21			113	**205**
	0.027	20			73	**103**

Biological Procedures

Unless otherwise specified experimental groups consisted of three female rats, 22–26 days of age at the beginning of the experiment. Daily injections were made on 3 consecutive days.

Standardization of gonadotropic substances was always made by subcutaneous injection. When testing for synergism the *in vitro* mixture of the two components was likewise injected subcutaneously. The desired amounts of prolan and synergic factor were dissolved in a total of 11 cc. of water; each rat received a total of 3 cc. of the mixture. Parallel tests were always made with each component of this mixture; each substance was dissolved in 11 cc. of water so that the total volume of liquid administered in control experiments and in the synergism experiments was the same.

In order to demonstrate antagonism, the hypophyseal fraction was always administered intraperitoneally, and the prolan was simultaneously injected subcutaneously. Each component was dissolved in 5.5 cc. of water, with daily injec-

tion of 0.5 cc. of each solution per rat. In the control experiments each of the components used in combination was dissolved in 11 cc. of water and 1 cc. was injected daily. As in the combination experiment the prolan was injected subcutaneously and the hypophyseal component intraperitoneally.

All experimental animals were sacrificed 96 hours after the first injection unless otherwise specified. The ovaries were observed under a binocular microscope, then carefully dissected and weighed. The weights of ovaries given in the tables are averages based on three animals.

Preparation of Fractions Potent in the Synergic Substance

The aqueous alcohol extraction method previously described (1) has been found to be the most satisfactory method of preparing comparatively potent fractions of the synergic principle. Frozen sheep anterior pituitary glands (1370 gm.) were ground very fine into 5000 cc. of 60 per cent alcohol. The mixture was allowed to stand for 6 hours at room temperature with occasional stirring after which the solvent was decanted and filtered and the fluid saved (*ca.* 4500 cc.). The gland residue was reextracted with 5000 cc. of 40 per cent alcohol for 15 hours. The solvent was again decanted (*ca.* 4500 cc.) and the extraction repeated with 5000 cc. of 40 per cent alcohol for 3 hours. The combined filtrates (14,000 cc.) were poured into 32,000 cc. of alcohol and a small amount of saturated alcoholic sodium acetate was added to precipitate the active material. By this means there was obtained 6.0 gm. of a powder, almost completely soluble in water and potent in 0.136 mg. dose (R3372, Table VII).

Isoelectric precipitation of the above product eliminated inactive material and other impurities. For this procedure 1 gm. of the substance was dissolved in 100 cc. water and the insoluble material centrifuged off. The solution was adjusted to pH 4.4 and a large proportion of the material then precipitated. The mixture was allowed to stand 8 hours at room temperature and the precipitate was centrifuged off. Most of the activity was retained by the supernatant liquor which was poured into 500 cc. of alcohol and the active material precipitated by the addition of a small amount of saturated alcoholic sodium acetate. The yield was 0.16 gm. of a powder, potent in 0.027 mg. dose (R3500, Table VII) and giving a positive biuret test.

For the study of the action of enzymes on the anterior pituitary, with particular reference to the synergic and antagonistic principles, an alkaline extract of anterior lobe was used as stock material. Desiccated sheep anterior hypophysis (20 gm.) was extracted thoroughly with 1000 cc. of dilute sodium hydroxide solution (pH *ca.* 8–9) and the insoluble material centrifuged off (the yield of the insoluble fraction was 10.5 gm. and contained little of the synergic factor). The alkaline solution was adjusted to neutrality and used as the starting point for the following digestions. It contained about 1 per cent of the anterior lobe material.

The digestions with trypsin were carried out by adding 1 part of trypsin (Fairchild Bros. and Foster) to 100 parts of anterior lobe. The solutions were adjusted

to pH 8.5 and kept at 37°C. throughout the digestion. The progress of the digestion was followed by determining the change in total acidity (10, 11). Aliquots of the solution were titrated at intervals with sodium hydroxide solution using thymolphthalein as the indicator. Digestion was found to be practically complete in 4 to 5 hours. Since no foreign buffer was used to maintain the hydrogen ion concentration, the solution was readjusted to pH 8.5 every 2 hours. The tryptic hydrolysis did not affect the synergic principle but digested much of the inactive protein present in the solution (Table VI).

Anterior lobe solution digested with trypsin was then subjected to digestion with erepsin. Erepsin (The Arlington Chemical Co.) was added in an amount equal to 1 per cent of the original concentration of anterior lobe, the solution adjusted to pH 8.0 and kept at 37°C. during the digestion. There was little change in titratable acidity during 4 hours but biological assay of the resulting solution indicated that the antagonistic factor had been destroyed with little harm to the synergistic principle (Tables III and VI).

Pepsin digestion of the alkaline extract of anterior lobe was carried out using 1 part of pepsin (The Wilson Laboratories) to 100 parts of anterior lobe material. Digestion at pH 4.0 and 37°C. completely destroyed the activity of the synergic principle within 4.5 hours (Table VI).

Solutions of the anterior hypophysis digested with trypsin and erepsin as described above gave no precipitate on addition of alcohol to 80 per cent concentration. The solutions were, therefore, first concentrated *in vacuo* (at a water bath temperature of 55°C.) to one-tenth the original volume. This concentrate on precipitation with four volumes of alcohol gave a product which was easily soluble in water and contained most of the activity present in the original solution. It was potent in 0.136 mg. dose and gave a positive biuret test.

SUMMARY

The hypophyseal substance—the synergic factor—which gives increased gonadotropic effects when combined with prolan has been shown to itself possess a definite though slight gonadotropic activity. It produces transitory follicular enlargement within 24–48 hours, which, however, regresses at once on cessation of treatment so that by the end of the 4th day the ovary again approximates its infantile weight.

The synergic principle indeed provokes only moderate ovarian development when administered in 100 times the dose necessary to demonstrate the activation phenomenon.

By means of isoelectric precipitation or by means of tryptic and ereptic digestion, fractions containing the synergic principle were freed of many contaminants, in particular of the antagonistic factor.

The synergic principle has been shown to be unaffected by digestion

with trypsin or trypsin followed by a short digestion with erepsin, but the action of pepsin inactivated the material.

A preparation of the active substance has been obtained which is potent in a total dose of 27 *gamma*.

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THE RECOGNITION AND COMPARISON OF PROLAN AND PROLAN-LIKE SUBSTANCES*

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It has been shown that the limited gonadotropic effect of prolan can be greatly enhanced by the simultaneous injection of a synergic principle from the hypophysis (1, 2). This suggested that the method could be used to demonstrate the presence of prolan or prolan-like substances occurring in blood or urines in conditions other than pregnancy.

The occurrence of a gonad-stimulating substance in the urine of men is manifested in certain pathological conditions (castration, genital carcinoma) (3, 4), but no evidence for the presence of such a substance in normal male urine has ever been obtained. In the course of this study, various methods for the concentration of prolan have been applied to male urine[1] and in the best concentrates (alcohol-ammonia extracts) it has been possible to demonstrate the presence of a prolan-like substance by administering it alone. Further, when these concentrates were strengthened by the addition of the synergic factor from the hypophysis, a significant augmentation of the infantile rat ovaries was elicited. This was the case with both the crude alcohol precipitate and the more concentrated extract (Table I).

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[1] We are grateful to Dr. J. A. Morrell of E. R. Squibb and Sons for generous supplies of a concentrate from urine of normal men.

It is a matter of no little concern to the physiologist that prolan has been found almost solely in the serum and urine of primates. With the sensitive synergism test at hand, it seemed important to inquire if small amounts of prolan-like substances could not be detected in the sera and urine of non-primates. Evidence to the effect that gonadotropic substances do in fact occur in mammals other than primates

TABLE I

Response of the Immature Female Rat to a Combination of the Hypophyseal Synergic Principle with Prolan-Like Substances from Human Sources

Prolan-like substance				Synergic principle (pig)		Combination* (*in vitro*)	
Source	Total dose in 3 days	Weight of ovaries	Description of ovaries	Total dose in 3 days	Weight of ovaries	Weight of ovaries	Activation
	mg.	*mg.*		*mg.*	*mg.*	*mg.*	*per cent*
Pregnancy serum, acetone-ammonia extract R2438	54.4	36	2–6 corpora	2.7	25	82	**90**
	27.3	26	2–6 "			57	**72**
Placenta, alcohol precipitate of aqueous acetone extract R2384	54.4	20	Large follicles	2.7	20	94	**327**
	27.3	17	" "			38	**90**
Normal male urine (age 20–40 yrs.)							
Crude alcohol precipitate R2666	272	12	Infantile	2.7	32	66	**106**
Reprecipitated from alcohol R2346	163	15	"	2.7	17	54	**200**
Alcohol-ammonia extract R2662	272	16	3–8 corpora	2.7	32	81	**155**
Alcohol-ammonia extract R2442	215	28	3–4 "	2.7	25	53	**51**

* The given weights of the two components were mixed *in vitro* and injected subcutaneously, daily, on 3 successive days with autopsy after 96 hours (1, 2). Three rats were used in each group.

has accumulated from parabiotic studies (Matsuyama (5)). Martins (6) and Emery (7) found by direct injection that the blood of the castrate male and female rat contains a gonadotropic substance. The sera of normal males and pregnant or non-pregnant females gave only a negative reaction as did the urines of all animals studied. Jeffcoate (8) found that gonadectomized rabbits excrete a gonad-stimulating substance in their urine.

Rats afforded the most convenient material for the present study and injection of sera or urinary concentrates from normal males and females, castrate and cryptorchid males and pregnant females all gave a negative reaction. By means of the synergism phenomenon,

TABLE II

Demonstration of a Prolan-Like Substance in the Urine and Blood Serum of Rats

Prolan-like substance injected in female rats (3 days)					Injected in male rats (10 days)†		
Source	Dose	Weight of ovaries	Combination with synergic factor* (*in vitro*): Weight of ovaries	Combination with synergic factor* (*in vitro*): Activation	Dose	Weight of seminal vesicles	Weight of testes
	cc.	*mg.*	*mg.*	*per cent*	*cc.*	*mg.*	*mg.*
Castrate rat blood serum‡ R3555	3	18	77	**320**	10	10	540
Cryptorchid rat blood serum‡ R3561	3	12	46	**150**	10	10	509
Normal female rat blood serum R3557	3	14	65	**260**	10	8	470
Pregnant rat blood serum R3558	3	14	58	**220**	10	10	447
Normal male rat blood serum R3559	3	20	41	**105**	10	9	520
(Controls)					—	(9)	(446)
	mg.				*mg.*		
Castrate rat urine, alcohol precipitate R3189	136	12	62	**244**	270	7	376
Cryptorchid rat urine, alcohol precipitate R3191	88	10	61	**238**	270	8	232
Pregnant rat urine, alcohol precipitate R3040	161	15	37	**105**			
Normal female rat urine, alcohol precipitate R3467	137	18	48	**150**			
(Controls)					—	(10)	(392)

* Synergic factor: dose, 1.36 mg.; weight of ovaries, 18 mg.

† As routine, six male rats, 21–25 days of age were used in each group.

‡ Male rats were rendered cryptorchid or castrate 3 weeks previous to the collection of their urines and sera.

however, it was possible to demonstrate the presence of a prolan-like substance in each of the above cases. These results are summarized in Table II.

The widespread distribution of prolan-like substances in all types

TABLE III

Demonstration of a Prolan-Like Substance in the Urine and Blood Serum of Various Non-Primates

Prolan-like substance			Synergic factor		Combination (*in vitro*)		Prolan-like substance in male rats		
Source	Total dose in 3 days	Weight of ovaries	Total dose in 3 days	Weight of ovaries	Weight of ovaries	Activation	Total dose in 10 days	Weight of seminal vesicles	Weight of testes
		mg.	*mg.*	*mg.*	*mg.*	*per cent*		*mg.*	*mg.*
Pregnant cow blood serum R2668	3 cc.	16	2.7	22	78	**254**			
Normal cow blood serum R3459	3 cc.	13	1.36	18	73	**305**			
Pregnant cow blood serum, acetone precipitate R2632	272 mg.	15	2.7	22	57	**159**	455 mg.	11	521
Pregnant pig blood, alcohol precipitate R3565	109 mg.	16	1.36	20	41	**105**	10 cc. (serum)	10	457
Normal female pig blood, alcohol precipitate R3566	109 mg.	18	1.36	20	68	**240**	10 cc. (serum)	7	563
(Controls)								(9)	(446)
Pregnant guinea pig urine, alcohol precipitate R3491	Equivalent of 41 cc. urine	16	1.36	18	64	**255**			
Normal female guinea pig urine, alcohol precipitate R3493	Equivalent of 30 cc. urine	13	1.36	18	31	**61**			
Pregnant dog urine, alcohol precipitate R3495	Equivalent of 35 cc. urine	14	1.36	22	34	**54**			
Pregnant rabbit urine, alcohol precipitate R3477	Equivalent of 75 cc. urine	17	1.36	22	39	**77**			
Normal female rabbit urine, alcohol precipitate R3473	Equivalent of 80 cc. urine	17	1.36	22	27	**0**			

of rats led to a similar investigation of other animal forms. The findings in the rat were largely confirmed. Serum of pregnant and non-pregnant cow,[2] pregnant and non-pregnant pig and urines of pregnant and non-pregnant guinea pig and pregnant dog were all negative when injected alone and all gave activation when combined with the synergic factor. The urine of pregnant and non-pregnant rabbits,[3] however, showed but slight activation when tested similarly and the urine of pregnant mares, none at all. These results are summarized in Table III.

Since the activation phenomenon has been observed in many instances regardless of whether or not the prolan-like substance was positive by itself, it seemed worthwhile to give subminimal doses of prolan from pregnancy urine with the synergic factor. In Table IV is shown the effect of combining varying doses of prolan with a constant amount of the hypophyseal component. It was rather surprising to find that pregnancy prolan was potent in doses lower than those which showed the activation phenomenon. It is, therefore, apparent that the prolan-like material present in most non-pregnancy urine is different from that in pregnancy.

Because of the theoretical interest attached to the possible origin of prolan and prolan-like bodies from the hypophysis it seemed important to determine whether or not gonadotropic substances when actually injected into rats would reappear in their urine. Ehrhardt has shown (11) that the urine of a non-pregnant woman contained prolan shortly after she had received a blood transfusion from a pregnant woman.

In this study prolan and pregnant mare's serum were injected into separate groups of rats and their urine was collected, concentrated and assayed. Rats injected with prolan secreted at least a portion of it in their urine (quantitative recovery was not attempted). Both the native urine and the concentrates gave positive reactions. Thus 6 cc. of the sevenfold concentrated urine gave 62 mg. ovaries. However,

[2] Leonard (9) has presented data that indicate the presence of prolan-like substances in the urine of pregnant and non-pregnant cows. Combination of the urinary product with the follicle stimulator (10) (which contains the synergic principle) gave increased ovary weights.

[3] We desire to thank Drs. Wade Brown and Louise Pearce for placing rabbits of known sexual history at our disposal.

urine from rats receiving pregnant mare's serum could not be differentiated from urine of uninjected controls. *This is in agreement with the fact that no gonadotropic hormone is found in the urine of the* pregnant mare.

Realizing that the physiological mechanism of the rat differs from that of primates in the handling of gonadotropic substances, we injected *rhesus* monkeys intravenously with pregnant mare's serum and hypophyseal gonad-stimulating substance (pig flavianate preparation). The urines were collected, concentrated and assayed in immature

TABLE IV

Effect of Combination of Varying Doses of Prolan with a Constant Amount of the Synergic Principle from the Hypophysis

Prolan (R3082)			Synergic principle (pig)		Combination (*in vitro*)	
Dose	Weight of ovaries	Description of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
mg.	*mg.*		*mg.*	*mg.*	*mg.*	*per cent*
13.6	43	Large follicles and corpora			155	**237**
6.8	44	" "	1.36	21 (Small and medium follicles only)	128	**172**
2.7	30	" "			75	**127**
1.36	32	" "			60	**71**
0.54	29	Few corpora			37	**15**
0.27	15	Infantile			26	**23**

female rats. A gonadotropic hormone was abundantly demonstrated in the urine of the monkeys injected with the hypophyseal hormone. An ovary weight of 62 mg. was elicited by the injection of a total dose of 69 mg. of the urinary concentrate. Combination with the synergic substance did not further increase the ovary weights. The urinary product from the monkeys injected with pregnant mare's serum contained no gonadotropic substance. The results were, therefore, comparable to those in the rat. Further, the urine did not show the activation phenomenon on combination with the synergic principle. The urine from normal controls did not cause ovarian development when injected alone or in combination with the synergic factor.

Since the synergic principle from the hypophysis has such a pronounced effect on the ovaries when combined with prolan it was of interest to see if there was a similar effect on the seminal vesicles.

TABLE V

Response of the Immature Male Rat to a Combination of the Synergic Principle from the Hypophysis with Prolan from Pregnancy Urine

Material injected	Injected 3 days, sacrificed 5th day, R1989			Injected 11 days, sacrificed 12th day, R2276			Injected 19 days, sacrificed 20th day, R2658		
	Dose	Weight of seminal vesicles	Weight of testes	Dose	Weight of seminal vesicles	Weight of testes	Dose	Weight of seminal vesicles	Weight of testes
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Prolan	27.3	33	471	110	85	610	180	125	900
Synergic principle	27.3	7	267	11	7	395	18	26	805
Prolan plus synergic principle		49	535		119	749		372	1362
(Controls)		(8)	(308)		(6)	(498)		(13)	(483)

TABLE VI

Response of the Immature Male Rat to a Combination of the Synergic Principle from the Hypophysis with Prolan-Like Substances from Human Sources

Prolan-like substance				Synergic principle		Combination (*in vitro*)			Controls	
Source	Total dose in 10 days	Weight of seminal vesicles	Weight of testes	Total dose in 10 days	Weight of seminal vesicles	Weight of testes	Weight of seminal vesicles	Weight of testes	Weight of seminal vesicles	Weight of testes
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Pregnancy urine R2276	110	85	610	11	7	397	119	749	6	492
Pregnancy serum R3066	90	54	689	4.5	15	730	41	785	9	444
Normal male urine R2277	660	6	365	11	7	397	7	613	6	492

This was found to be the case: The synergic principle itself had little or no effect, but in combination with pregnancy prolan it caused a definite increase in the development of the seminal vesicles (Table V). These results also indicate that the 10 day test is adequate for the attainment of significant values.

The action of prolan-like substances from various sources on the testes and seminal vesicles is shown in Table VI and also in Tables II and III. It is perhaps significant that all of the non-primate sources, shown to contain a prolan-like substance as evidenced by the activation phenomenon with immature females, were uniform in their lack of effect on the testes and seminal vesicles. Normal male urine conformed to the above classification.

SUMMARY

The synergism phenomenon has made possible the recognition of substances, which we have called "prolan-like," in a wide variety of conditions. Indeed, it has been possible to demonstrate a prolan-like material in the urine of normal men.

The method has shown a wide distribution of prolan-like substances in sera and urines of non-primates without demonstrating, however, significant difference in the reactions secured from pregnant as contrasted with non-pregnant states, or in males as contrasted with females.

The synergism phenomenon with pregnancy prolan can also be shown in the increased development of the seminal vesicles of immature male rats. However, such males cannot be advantageously employed in the detection of prolan-like gonadotropic substances occurring in the blood and urine in conditions other than pregnancy, for the prolan-like substances usually do not effect appreciable development of the seminal vesicles.

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CONCENTRATION OF THE GONADOTROPIC HORMONE IN PREGNANT MARE'S SERUM*

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In 1930, Cole and Hart (1) discovered the presence of a gonadotropic hormone in the blood stream of mares in early pregnancy. It was detected almost simultaneously by Zondek (2). Cole and Hart established the following facts. The hormone is present in the period beginning at 37 days after the fertilizing coitus and lasts until approximately the 175th day but the presence of very appreciable amounts of it is limited to the period between 43 and 80 days. The period of greatest concentration occurs apparently somewhere in the interval between the 50th and 65th day and the native unaltered serum drawn from the mare's jugular vein at this time is capable of exerting clear gonadotropic effects in immature (21–26 day old) female rats or mice within a hundred hours following six doses of 0.005 cc. These investigators and others associated with them have continued study of the very interesting hormonal and tissue changes in pregnant mares and in the horse fetus (3–7). They have shown that during the period of high concentration of gonadotropic hormone in the mare's blood stream, its ovaries exhibit the formation *de novo* of many corpora lutea, an unique phenomenon; and that in the period immediately subsequent thereto, the fetal gonads exhibit an astonishing hypertrophy caused by the massive appearance there of interstitial cells.

It is apparent that equine hormonal and tissue conditions are fully as remarkable as those which characterize primates (with the Aschheim-Zondek reaction of pregnancy blood and urine) and that further inquiry here will be well repaid. Evans, Meyer and Simpson (8) included the hormone from the mare's blood in their comparative study of gonad-stimulating substances from the hypophysis and other

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sources and showed the relatively great concentration of gonadotropic hormone in this source.

The chemical properties of the gonadotropic substance of pregnant mare's blood have been studied by Goss and Cole (5) and by Cole, Guilbert and Goss (6). They found it possible to concentrate the serum at 36°C. in partial vacuum without appreciable loss of potency. *Indeed, heating the serum to its coagulation point and even to the temperature of the boiling water bath for several minutes failed to destroy the gonadotropic principle.* The active material failed to dialyze through a collodion membrane and could not be detected in ultrafiltrates. No effect was produced when massive doses of serum were fed to rats.

In agreement with Cole, Guilbert and Goss (6) we have found the active principle to be much more stable to alkalies than to acids at ordinary temperatures. No loss of potency could be observed in samples which had been allowed to stand 15 hours at 22°C. in range pH 3.7 to pH 11.0. Below pH 3.7 the loss of potency was distinctly apparent and potency decreased with increasing acidity.

Pepsin had little effect on the gonadotropic substance when the digestions were carried out at pH 4–5 at 37°C. for 4 hours. At pH 1.8–2.0 the activity was destroyed but this may not have been due to the action of pepsin since controls in which no pepsin was used were also inactivated at this pH and temperature.[1] Trypsin at pH 8.5 inactivated the hormone after 4 hours at 37°C. The products of peptic and tryptic digestion did not increase the gonadotropic effect of pregnancy prolan.[2] Inactivation was also observed when solutions at pH 7.5 were heated for 4 hours at 60–80°C. and the resulting product showed no tendency to increase the gonadotropic effect of pregnancy prolan.

When the acetone-dried serum was dissolved in anhydrous formic acid for 2 hours at 25°C. and the serum proteins recovered by precipitation with acetone, it was found that the gonadotropic activity had been lost and no increase in the activity of pregnancy prolan was observed when the inactivated material was injected with it.

The crude serum of the pregnant mare shows a very considerable

[1] Cole, Guilbert and Goss (6) found partial inactivation of the hormone followed peptic digestion at pH 3.

[2] *Cf.* Evans, H. M., Simpson, M. E., and Austin, P. R., *J. Exp. Med.*, 1933, **57**, 897.

gonadotropic potency. Cole and Hart (1) found the maximum ovarian response in rats followed 6 injections of 0.2 cc. of serum from a mare 78 days pregnant, autopsy being performed 96 hours after the first injection. Increasing the dose to 0.5 cc. showed no further increase in effect, while as has been mentioned, as little as 6 injections of 0.005 cc. of crude serum produced ovarian effects which could be detected by histological section.

Goss and Cole (5) attempted to concentrate the active principle by fractional precipitation of the serum with salts. They found that the fraction precipitable between the concentrations 20 and 27 per cent sodium sulfate contained the greater part of the activity while only 11 per cent of the serum protein was found in this fraction. Evans, Meyer and Simpson (8) prepared acetone-ammonia extracts from the acetone-dried serum and precipitated solutions of these acetone-ammonia extracts with flavianic acid. These procedures gave potent materials still contaminated by inactive serum protein.

With the hope of finding a better method of attack on the problem of the isolation of the active agent we have made a comprehensive study of the behavior of the hormone towards a number of adsorbents following the technique which has yielded conspicuous success in the purification of enzymes by Willstätter and his school. This study has shown that aluminum hydroxide preparations are excellent adsorbents for the gonadotropic substance found in the serum of the pregnant mare.

When aqueous solutions of the acetone-dried proteins of pregnant mare's serum were adjusted to pH 3.5 and treated with suspensions of aluminum hydroxide (Willstätter Type A and Type B) (9) it was found that the active substance was readily adsorbed on the surface of the aluminum hydroxide. In this way it has been possible to separate the active agent from a large part of the inactive protein material. Subsequent washing of the aluminum hydroxide on which the hormone had been adsorbed, using acetate buffer at pH 3.5 or water, removed practically none of the active material. The elution of the active substance was, however, easily accomplished by dilute ammonia or by converting the aluminum hydroxide into the basic phosphate with disodium phosphate solution in the usual manner. The active solutions

obtained by elution were freed from salts by dialysis in Visking[3] membranes and their potency and content of solid material determined.

In this way preparations were obtained which gave, in a total dose of 0.006 mg., an ovarian reaction in mice comparable to the maximum effect observed with pregnancy prolan. A description of typical experiments will make the procedure clear.

EXPERIMENTAL

Chemical Procedures

30 gm. of acetone-dried serum was dissolved in 6000 cc. of 0.005 N NaOH and the solution adjusted with dilute HCI to pH 3.5 using the glass electrode (10, 11). 300 cc. of a suspension of freshly prepared aluminum hydroxide Type B, containing 3 gm. of air-dried solid, was added and the solution vigorously stirred for 30 minutes. The aluminum hydroxide on which the hormone had become adsorbed was now collected by running the solution through a Sharples supercentrifuge until it came through perfectly clear. The cake of adsorbent was removed from the steel cylinder and shaken up with 100 cc. of molar acetic acid-sodium acetate buffer of pH 3.5 for 30 minutes. The suspension was centrifuged and the supernatant discarded. The precipitate was shaken up with 200 cc. of 0.5 per cent aqueous ammonia and the ammoniacal suspension allowed to stand at 5°C. overnight. The suspension was centrifuged and the supernatant decanted, chilled, brought to about pH 8.0 with dilute HCl, and dialyzed. The elution could be equally readily accomplished by the use of disodium phosphate solution instead of ammonia.

The superiority of the aluminum hydroxide suspensions as selective adsorbents for the hormone became apparent to us as a result of a careful study of a group of adsorbent materials and their applicability to the problem. Kaolin, kieselguhr, alundum, tricalcium phosphate, Lloyd's reagent, permutit, aluminum hydroxides Types A, B and C and talc were systematically studied in regard to their ability to adsorb the hormone. The aluminum hydroxides Types A and B proved by far the best adsorbents of the group. Aluminum hydroxide Type C was considerably less efficient. In very acid solutions Lloyd's reagent adsorbed an appreciable amount of the active material.

It was noticed that the effectiveness of the aluminum hydroxide preparations depended in an unusual degree on the pH of the hormone solution. The table illustrates this point very clearly. Solutions of the acetone-dried serum of pregnant mare's blood were adjusted to definite pH's, using the glass electrode, and were treated with the

[3] Regenerated cellulose tubes manufactured by the Visking Corporation, Chicago.

same amounts of aluminum hydroxide Type B. After adsorption the supernatants were tested for the presence of the hormone.

Average Weight of Ovaries of Mice Injected with Supernatants Left after Adsorption with Aluminum Hydroxide Type B

Dose	Hydrogen ion concentration						Controls
	pH 9.8	pH 8.5	pH 7.3	pH 4.7	pH 3 5	pH 2.2	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Equivalent to 14 mg. dried serum	15	14	15	11	3	19	11
Equivalent to 7 mg. dried serum	10	13	11	10	3	15	12
Equivalent to 1.4 mg. dried serum	5	3	3	3	2	3	4

Biological Procedures

In testing the potency of chemical fractions groups of 3 mice 6–10 gm. in weight and 21–25 days of age were injected on 3 successive days. Autopsy was performed 96 hours after onset of treatment. Ovaries were observed under a binocular microscope, dissected and weighed. The ovarian development taken as representative of any fraction was the average weight of pairs of ovaries in the group of 3 mice. All preparations were tested in a series of dilutions in order to determine maximal and minimal doses. The mouse gave sufficient ovarian response to this powerful gonadotropic hormone to make it a thoroughly satisfactory animal form for following the purification of the hormone from pregnant mare's serum. When giving the maximal response the ovaries weighed 12–15 mg. (After injection of the hormone found in the urine of pregnant women (prolan) the maximum ovary weight attained is only about 6–8 mg.) The infantile ovaries would weigh from 1–2 mg. Ovaries giving the minimal observable response weighed about 3–4 mg. The most potent product obtained by absorption gave the minimal ovarian response when given in a total dose per mouse of 0.003–0.002 mg. At the 0.006 mg. dosage the ovaries weighed 6–8 mg. (*i.e.* were as large as could be produced by any dose of prolan), and at a 0.012 mg. dose the maximum ovarian weight (12–15 mg.) attainable with this product was reached.

Cole, Guilbert and Goss observed the gonadotropic effects of pregnant mare's serum when injected into immature male rats (6). Accordingly, we have similarly studied the effect of the concentrated material. The preparation was injected for 10 and 20 day periods giving 5.5 mg. per rat per day. (This particular preparation gave the minimal response in female mice following injection of a total dose of

0.012 mg.) At the end of 10 days the testes weighed 1516 mg. and the seminal vesicles 200 mg. (the control organs weighed respectively 495 mg. and 8 mg.). After 30 daily injections the testes weighed 2620 mg. and the seminal vesicles weighed 979 mg. (control organs weighed 1155 mg. and 13 mg. respectively). Histologically it was found that after 10 daily injections the testicular tubules were already noticeably increased in diameter and that there was a marked increase in interstitial tissue. After 30 days the tubules were even larger, the spaces between tubules were densely packed with interstitial tissue and spermatozoa were found in the lumen of the tubules.

SUMMARY

The gonadotropic hormone of the blood of the pregnant mare has been greatly concentrated by adsorption on active aluminum hydroxide followed by elution. The preparations so obtained gave demonstrable gonadotropic effects within 100 hours in 21 day old female mice following three subcutaneous injections of 0.001 mg. in 1 cc. of physiological saline.

As is well known, other gonadotropic substances do not cause conspicuous development of the male gonads but injections of comparatively large doses of these preparations into immature male rats caused marked development of the testes, which in 10 days were trebled in weight. An astonishing increase in the weight of the seminal vesicles resulted, for these organs were approximately 75 times heavier than in controls.

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CONCERNING THE RELATIVE RESPONSE TO BLOOD GAINS AND BLOOD LOSSES; AND HABITUATION TO AN EXCESS OF BLOOD PIGMENT

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The experiments to be described were begun with the aim of determining the amount of blood, as red cells, formed each day in rabbits. It was assumed that if this amount were supplied from without the erythropoietic tissue would cease to work. Robertson (1) had noted that repeated large transfusions resulting in a superabundance of hemoglobin caused a practical disappearance of reticulated red cells from the circulation of rabbits, indicating that the marrow had become less active. When, after a time, the alien blood was suddenly destroyed as result of the formation of immune bodies by the recipient, a profound anemia developed, this fact as well as others showing that the transfused cells had functioned so effectively that the marrow had largely left off erythrocyte production. In some recipients no blood destruction was evident, and these formed no antibodies demonstrable on test. For the purpose of the present work such a state of affairs was essential. A measured small amount of compatible blood was introduced into the circulation each day, with the expectation that the marrow would lessen its erythropoietic activity in proportion as its task was taken over, the amount of strange blood necessary to induce it to cease work being presumably that which it would form each day under normal conditions. The results of the experiments have proved the primary assumption to be incorrect. No indication has been found that marrow activity lessens when small quantities of compatible blood are added to the circulation day after day. On the contrary, the marrow keeps on working and itself contributes to the increase in hemoglobin. Not only this, but when no more blood is introduced from without, and the superabundance of hemoglobin begins to diminish, the marrow becomes abnormally active, producing red

cells in such quantity as to maintain the abnormal state of affairs. An habituation of the organism to this state has come about.

There would appear to be no papers in the literature on the consequences of bringing about a superabundance of hemoglobin gradually by experimental means, though there are many describing the consequences of doing this abruptly, and many dealing specifically with the relation between marrow activity and hemoglobin concentration. Boycott and Oakley (2) have recently dealt with the latter theme comprehensively, in a paper appearing since the present work was completed. Interested primarily in the regulation of marrow activity, they undertook to stop it by supplying blood from without; but this they found themselves unable to do. The conditions were drastically altered in their experiments, and their findings differ in many respects from those now to be reported. Their data, considered in parallel with our own, provide numerous enlightening contrasts. They discuss in detail some of the problems of marrow activity and for these, as for not a few other pertinent matters, it has seemed well to refer the reader to their paper rather than to attempt to recapitulate what has been admirably summed up.

The Effects of Gradual Additions to the Blood

A rough, first test was made to find how much blood must be introduced daily into the circulation of rabbits in order to cause the erythropoietic tissue to stop working. As a criterion of stopping work the disappearance of reticulated red cells was looked for; and at the end of the series of transfusions the red marrow of some of the animals was examined for its content of these cells. A group of six normal rabbits were given transfusions of whole compatible blood, 6 days in 7, the daily amounts being $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, 3, and 4 cc. for the respective animals.

An extensive literature shows that the number of reticulocytes in circulation varies with the erythropoietic activity; and dependence is now justifiably placed upon the count as indicative of what is occurring in the red marrow. Boycott and Oakley discuss these points at length and they have succinctly stated the general conclusion of investigators:—" . . . while changes in the proportion of reticulocytes are a good index of changes in marrow activity they are not a directly quantitative measure of it." Robertson (1) found that the marrow of rabbits maintained in plethora by the injection of large amounts of blood showed but few reticulocytes as compared with the normal content.

The rabbits, normal animals of mixed breed from stock, weighed from 1200 to 1550 gm. at the beginning of the transfusions and from 1600 to 2100 gm. at their end. They were kept in separate cages and fed hay daily, with the addition three times a week of a mixture of equal parts of oats and commercial food pellets. Water was available to them at all times.

In this series we followed the method employed by Robertson (1), aspirating the blood directly from the heart into a syringe containing 1 cc. of a 1 per cent solution of sodium citrate in normal saline. Normal compatible donors were employed in rotation, each being discarded after two or three bleedings to the amount necessary for all of the transfusions of a day. The method of Rous and Turner (3) was employed in the tests for compatibility. For 20 days before the transfusions were begun and just prior to each of these latter, reticulocyte counts and determinations of the blood hemoglobin were made. During the preliminary period the amount of hemoglobin did not vary significantly, but in several cases (Charts 1 and 2) the number of reticulocytes increased gradually.

The blood samples for counts and hemoglobin determinations were regularly taken in the morning, before the feeding of the day. Both counts and determinations were done by the same person in all of the experiments. They were made on blood from the vein of an ear rendered hyperemic by contact with a bottle of warm water. For the hemoglobin determinations, 20 c.mm. of blood was mixed with 5 cc. of 0.1 N hydrochloric acid and allowed to stand at least 1 hour. The readings were made by means of a Duboscq colorimeter supplied with the yellow glass matching-disc of Newcomer (4). The findings are expressed in grams per 100 cc. of blood.

A white cell pipette was used for the reticulocyte counts. Blood was drawn up to the 1 division and diluted to 11 (1–10) with the staining mixture employed by Friedlander and Wiedeman (5). Staining took place for 15 minutes when a drop of the mixture was placed on a slide and counting was begun at once of the reticulocytes occurring among a thousand cells in uniform fields.

The eventual reticulocyte determinations on the red marrow were made in two ways. In one a small piece of marrow was smeared on a slide previously prepared by allowing a concentrated alcoholic solution of brilliant cresyl blue to dry upon it, thus leaving a film of the dye. Such preparations could be counterstained with Wright's stain and were useful in examining for other manifestations of bone marrow activity. The second method was to wash out a portion of the marrow by forced injections into it of normal saline through a hypodermic needle after the method of Robertson. The mixture thus obtained was spun and counts were made on the sediment diluted with staining mixture as in the case of the blood samples. The period of transfusion ranged from 30 to 70 days.

In only one of the six recipients did an evident incompatibility develop to mar the findings. In this animal, which received 3 cc. of blood each day with result that the hemoglobin percentage mounted rapidly, there occurred the characteristic phenomenon first described by Robertson; namely, a suddenly developing, pronounced anemia although the transfusions were continued. With the anemia there was associated an appearance of strong isohemagglutinins in the blood.

The circulating hemoglobin diminished from 11 gm. to 4 gm. per cent between the 7th day and the 14th day of transfusion, and then rapidly mounted again, the percentage of reticulocytes rising from 20 to 550 per thousand as repair took place. The animal will not be considered further.

No hemoglobin increase developed in the rabbit receiving ½ cc. of compatible blood per day during more than 1 month of transfusion, and its reticulocytes varied throughout within the limits of the pre-transfusion normal. In each of the other four rabbits, those receiving daily 1, 1½, 2, and 4 cc. of blood respectively, the hemoglobin percent-

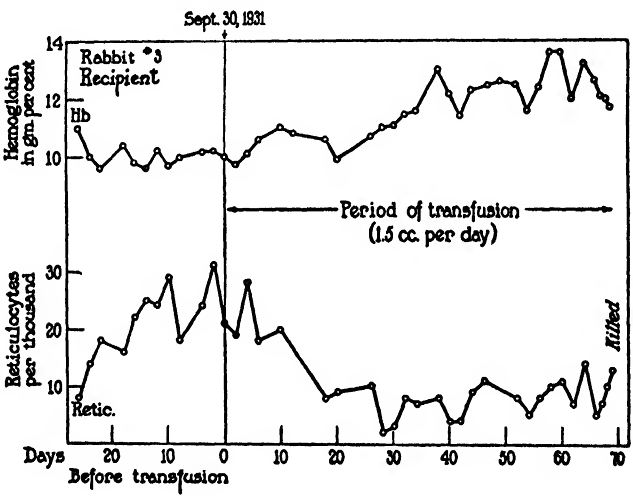

CHART 1

age increased,—gradually in the case of the rabbits receiving the smaller amounts of blood, promptly and greatly in the one injected with 4 cc. In all these instances some depression of marrow activity occurred as evidenced by a drop in the reticulocyte percentage; but it did not fall enduringly below the level at the beginning of the pre-transfusion period, and at autopsy the marrow count of reticulocytes was not significantly different from the normal, great numbers of these cells being present. Charts 1 and 2 illustrate the findings.

As already stated, the animals serving as recipients were "normals" selected from stock. It seemed possible that their initial amounts of

hemoglobin (9 to 10 gm. per cent) might have been near the lower limit for normality, and that the hemoglobin increase, after the smaller transfusions at least, might not have constituted a superabundance of the pigment but have been only a natural betterment consequent upon unusually favorable conditions for blood production. The rabbits were still growing rapidly, which introduced another variable. In the attempt to control conditions more strictly, a new group of animals was studied, adults selected as having large initial quantities of hemoglobin in the circulating blood. Two had 12 and 13 gm. per cent, and these received every day for 35 days without exception ½ cc.

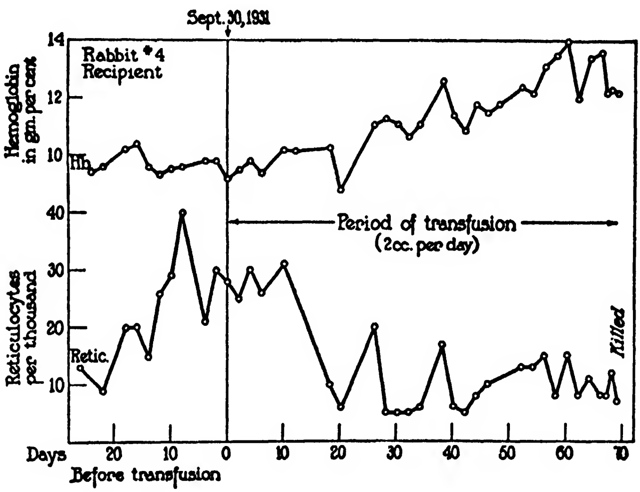

CHART 2

of compatible blood; while three other animals with 11, 12, and 12.1 gm. per cent of hemoglobin respectively were given 1 cc. *per diem.* The weights ranged from 1880 to 2280 gm. at the beginning of the transfusions and from 2100 to 2450 gm. at their end. The same technique of transfusion was employed as in the previous experiment.

The observations on the two animals first mentioned were marred by intercurrent illness (snuffles) in one case and by the development of antibodies against the alien blood in the other; but these complications did not develop until after a progressive increase in hemoglobin had occurred as result of the transfusions. Despite this increase no drop in the reticulocytes took place. In the rabbits receiving 1 cc. of blood each day for 35 days there were more considerable increases

in hemoglobin, to as much as 14 gm. in one case; yet the reticulocyte percentage, though touching zero on a single occasion in one of the animals, underwent no enduring, significant reduction.

The results in this series confirmed the previous observations. Even in rabbits with what appeared to be an abundance of hemoglobin, the introduction from without of 1 cc. of blood each day caused a considerable increase in the amount of the pigment per 100 cc. of blood. Yet

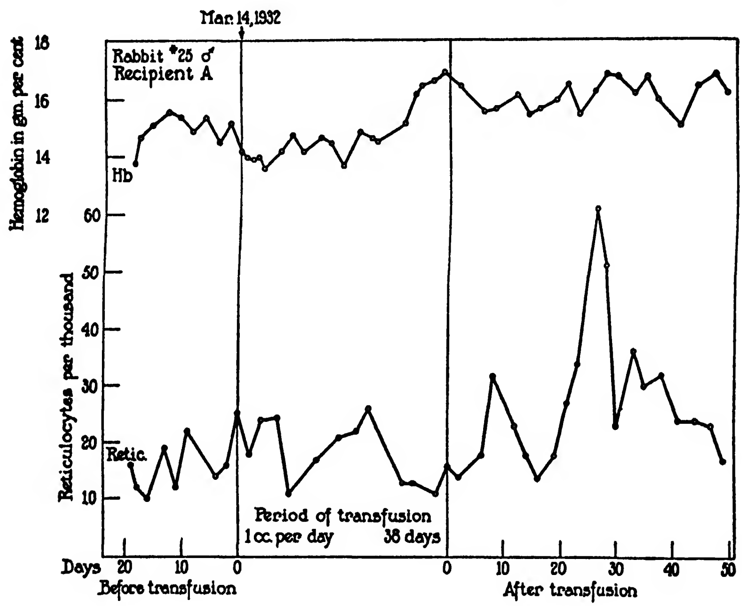

CHART 3

reticulated cells continued to circulate in good numbers, whence one might infer that there was no considerable marrow depression. The findings will not be recorded in detail since better controlled and far more convincing evidence was obtained in the next group of animals.

For the purposes of this experiment, adult rabbits were selected, both as recipients and as donors, that had notably great amounts of circulating hemoglobin,—far beyond the average "normal" quantity. A single donor was provided for each recipient in order to narrow the chances that the introduced blood might have a hidden incompatibility;

and frequent tests were made to be sure that one had not developed. In the experiments of Robertson (1) as also in Rous' (6) study of induced auto-antibodies, incompatibility leading to destruction within the organism of transfused blood was regularly accompanied by an agglutination *in vitro* of the donor's corpuscles by the recipient's serum; while in those instances in which isoagglutinins were lacking no such destruction was evident. To make sure of obtaining wholly com-

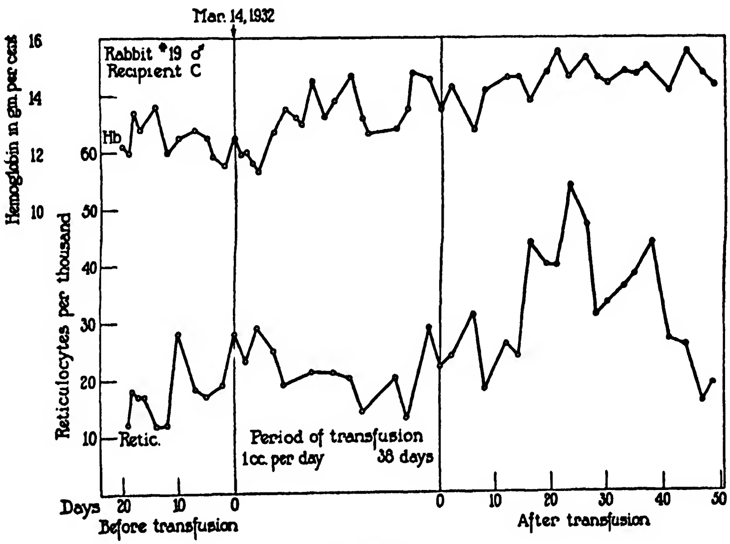

CHART 4

patible donors, especially searching agglutination tests were now made. As a check upon the general findings one recipient was purposely given incompatible instead of compatible blood.

The donors were vigorous animals which it was supposed would easily bear the loss of 1 cc. of blood *per diem*, as indeed proved to be the case. The condition of their blood was studied with the same care as was that of the recipients. Frequent hematocrit determinations of the red cell bulk were made in both groups. The observations were kept up for weeks after the transfusions had been discontinued.

Five compatible pairs of animals and one incompatible pair were employed. For the selective agglutination tests, the serum of the recipients was mixed with the washed cells of the donors, 9 parts of serum to 1 of a 50 per cent suspension of

the cells in salt solution. After 2 hours at room temperature, the mixtures were examined in the gross and microscopically. There was, in the case of the incompatible pair, well marked gross and microscopic agglutination of the donor's cells. As an accessory check upon the development of incompatibility during the transfusions, specimens of the blood of the recipients were examined for auto-agglutination (7). None transcending the normal was found save in the case of the animal receiving incompatible blood.

The initial weights of the rabbits were from 1800 to 2400 gm., and they were weighed each week, the figures showing a steady, gradual gain, as great in the donors as in the recipients, the final range being from 2300 to 3200 gm. All were kept in individual cages and on the same diet as the preceding series, save that cabbage was given three times a week.

The hemoglobin percentage, the number of reticulocytes, and the red cell bulk were ascertained at frequent intervals, usually every other day, for a period of at least 20 days preceding the first transfusion; and the observations were kept up during the 37 days of transfusion and for 6, in some cases 8, weeks thereafter. The blood specimens were taken in the morning, prior to feeding, the transfusions being done between 11:30 and 12 noon.

No anticoagulant was employed in this series to keep the injected blood fluid. The donor was placed in a covered box from which its head projected and the recipient in another by its side on the laboratory table. The shaved and oiled ear of the donor was heated until an active circulation had developed. A small cut was made in one of the marginal veins of the ear, and as the blood gushed forth it was steadily drawn up into a tuberculin syringe. When 1 cc. had been obtained in this manner, all further bleeding was prevented by an assistant and the injection into the ear vein of the recipient was quickly made. In this way no time was lost, the interval from the nicking of the donor's ear vein to the completion of the transfusion averaging not more than 60 seconds. Such care was taken for hemostasis that usually the donor lost no more than the desired 1 cc. of blood, at most not more than a drop. It has been found possible to extend the method to the transfusion of larger amounts; but when these exceed 4 cc. the risk of clotting becomes great. The first 2 or 3 cc. are much more rapidly obtainable than larger amounts, owing to contraction of the vessels of the bleeding ear. This caused trouble in some later experiments. For the determinations of red cell bulk the Van Allen hematocrit was used, with normal saline as the diluting fluid. Care was taken so to prepare this latter that changes in cell bulk due to osmotic imbalance were excluded. The findings during the transfusion and after periods showed that the red cell bulk varied directly with the hemoglobin quantity, as one might have expected under the conditions. Hence they are only occasionally charted.

In the analysis of the charts the experiment can be divided into three stages. There was the control period after the animals had been selected from stock, during which they lived under the same conditions as obtained subsequently when they

functioned as donor and recipient. Then followed a period of 37 days, on each of which the recipient received from his paired donor 1 cc. of whole blood. And there was a post-transfusion period of observation ranging from 40 to 50 days (see Chart 3, Rabbit 25). The preliminary hemoglobin amount of the recipients ranged from 12 gm. per cent to about 15 gm., averaging 13 gm. That of the donors had the same range but was slightly less, averaging about 12.4 gm. when the transfusions were started. They were not begun until the fact was plain that the amount of blood pigment was practically constant from day to day.

Charts 3 and 4 are typical of the findings in the group of animals receiving 1 cc. of compatible blood per day, an amount approximating 1/100 of their own initial quantity. There occurred in every instance (see Chart 5 giving averages) a progressive increase in hemoglobin percentage, gradual, as one would expect under

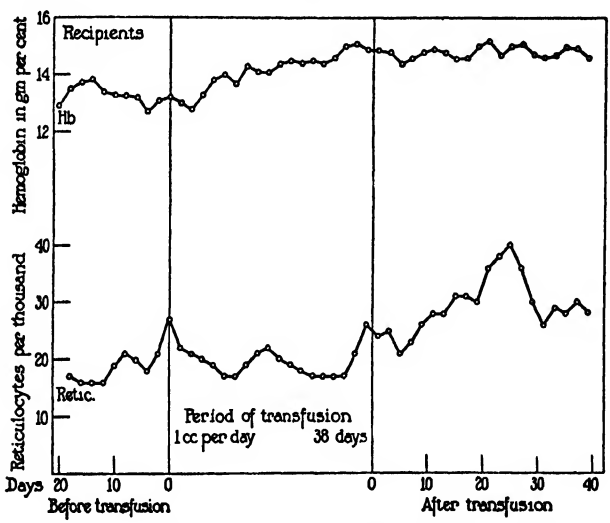

CHART 5. Composite chart of animals receiving small amounts of compatible blood daily.

the circumstances, but continuous, the average increase being about 2 gm. during the 38 transfusion days, the consequence being that sometimes the ultimate circulating amount far exceeded that encountered in any normal rabbit by the author. For example the rise in Rabbit 25 (Chart 3) was from an average of 15 gm. to one of nearly 17 gm. The reticulocyte percentage remained essentially unchanged throughout the transfusion period, a fact shown for the group as a whole by Chart 5.

Analysis of the Findings in the Recipients of Blood

The fact that the hemoglobin percentages rose markedly in the recipients does not necessarily mean that the pigment was present in

superabundance. The rises might conceivably have been the result of a diminished blood volume. But there are observations on record, notably the extensive ones of Boycott and his coworkers, which prove that after transfusion the blood volume returns to normal or is only slightly increased. A lessening in volume seems never to have been observed.

A second possibility has been mentioned already; namely, that the increase in hemoglobin was within the bounds of the normal, representing merely a blood betterment consequent upon favorable conditions. The experiment just described was designed to exclude this possibility, through the selection as recipients of rabbits having the highest initial amounts of hemoglobin encountered in normal animals. It is, of course, impossible to state precisely the upper limit of the normal, but it is sufficiently demarcated for present purposes by the data of Pearce and Casey (8), who themselves worked with Rockefeller Institute stock. The figures on blood hemoglobin that they obtained by the examination of 174 animals procured from stock range from 28 to 90 per cent (Newcomer, 4.7 to 15.2 gm. with the instrument Pearce and Casey employed); but pathological instances were not ruled out, a fact sufficiently obvious from the figures. The distribution frequency of the curve representing the individual findings, as also the data of a subsequent paper, yield a maximum and minimum range for the generality of animals of from 52 to 74 per cent (8.8 to 12.5 gm. per cent). The first group of "normal" animals of the present work had initial hemoglobin values of 9 to 11 gm. per cent and those of the third group from 12 to 15 gm. with an average of 13 gm. It is evident that prior to transfusion the first group of animals utilized as recipients had hemoglobin values somewhat above the lower level of the normal while those of the third group, which had been selected for high hemoglobin values, were at or near the normal maximum. In these latter animals a further considerable increase occurred during the transfusion period, one which in some instances definitely transcended the individual maximum encountered by Pearce and Casey. Such an increase can justly be regarded as constituting superabundance.

The mounting curves expressive of the hemoglobin percentages in the individual charts yield no indication of a "ceiling," such as could be taken to represent the upper limit of the normal, beyond which

obstacles to the increase in pigment might conceivably be encountered. In Rabbit 25, Chart 3, the curve followed the same slant in reaching 17 gm. per cent as in Nos. 1 and 2 in which it attained to only 13.8 gm. per cent and 14 gm. per cent respectively.

In what way did the increase in hemoglobin come about? There are several possibilities. One might suppose that the daily addition of blood from without was greater than that provided by the marrow and that this tissue ceased to work, a fact masked by the persistence in circulation of the reticulocytes already present or injected with the strange blood. But reticulocytes do not persist as such in the circulation. Boycott and Oakley (2) have reviewed the numerous papers which go to show that those of the rabbit mature into ordinary red cells in from 1 to 2 days after they leave the marrow. Those introduced with transfused blood disappear rapidly. Otherwise the count of such cells could not drop to zero as it frequently does for a brief period in rabbits receiving massive transfusions (1, 2). The daily introduction of 1 cc. of blood into the recipients of the present experiments would not suffice of itself to provide enough reticulocytes to maintain the count even if those introduced persisted as such throughout the transfusion period.

It is possible to calculate the total number of reticulocytes transfused in those instances in which their number was followed in the donors (Charts 3 and 4). For example in Recipient C, the reticuloycte count after 30 days when the hemoglobin had increased from 12.4 gm. per cent to 14.6 gm. per cent was still 3 per cent, about what it had been at the beginning. During this period the animal had received 30 cc. of strange blood from Donor C, carrying an average of 2.5 per cent of reticulocytes. This was introduced into a blood bulk of 113 cc. (since the rabbit has about 4.7 per cent of its weight in blood (9)). Assuming that all of the introduced reticulocytes persisted as such throughout the transfusion period, the gradual accumulation of them would account at most for only one-fourth the number in circulation at the end of the transfusion period. And the necessary assumption is not justified.

One is forced to conclude that the continued presence of reticulocytes in normal number throughout the transfusion period resulted from a persisting activity on the part of the erythropoietic tissue.

Granting that the marrow continued to work, to what was its work due—to lack of sensitiveness to the gradual increase in circulating blood pigment, as represented by the strange red cells, or to stimulation

resulting from the intercurrent destruction of these cells? Care had been taken to provide compatible cells, yet many of them must have been destroyed in the natural course of events, together with the cells of the host; and they might even have been destroyed practically at once after introduction, a fact concealed through the activity of the erythropoietic tissue to form new ones.

The total increase in circulating hemoglobin was far from being as great as it should have been had none of the introduced blood been destroyed and the blood volume remained constant. The average final weight of the five rabbits of Chart

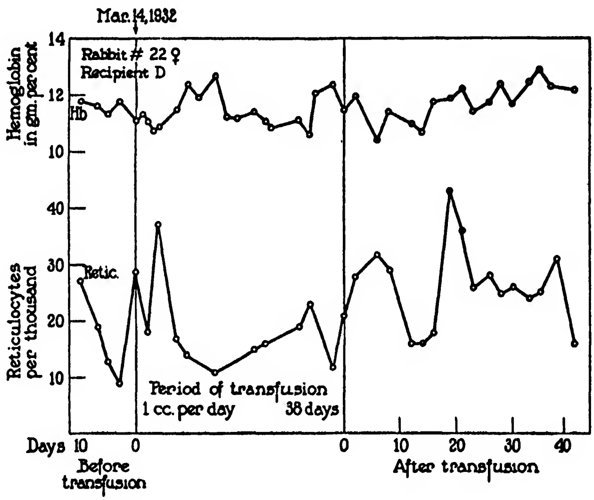

Chart 6. Effect of incompatible blood in small amounts.

5 was 2580 gm. Assuming that the proportion of the blood volume to the weight remained stationary, and that it amounted to 47 cc. per kilo, the total increase in hemoglobin at its height represented an accretion of only about 18 cc. of blood, whereas 37 cc. had been injected. Boycott and Oakley found that large transfusions never raised the hemoglobin to the figure it should have reached on calculation, a finding which may have been due in some small part to an increase in the total blood volume, as may that now under discussion. The daily wastage from normal blood destruction, as shown by the bilirubin output, varies directly with the quantity of red cells in circulation (10).

Boycott and Oakley have reviewed the contradictory literature dealing with the question of whether the products of blood destruction act to stimulate the marrow. The fact has many times been proved that when the body needs blood the introduction of materials that can be used in its formation, laked blood for ex-

ample, results in an increased activity on the part of the erythropoietic tissue. Indeed McMaster and Haessler (11) have shown that increases in the amount of this tissue to meet the emergency of anemia from hemorrhage are directly conditioned by the availability to it of the materials for blood formation. But increased reparative activity consequent upon the availability of such materials is not necessarily the same as direct marrow stimulation. Boycott and Oakley themselves found no such stimulation as result of the products of blood destruction save in a special instance, that of citrated, laked blood injected subcutaneously, laked blood as such failing of effect. Robertson did not obtain stimulation by transfusing incompatible blood to rabbits. As a check upon the possibility that in the present experiments the cells transfused were destroyed soon after introduction and utilized in the formation of new blood, agglutination tests of the third series of animals were made from time to time throughout the transfusion period to find whether the recipients had reacted against the donors' blood so that it had become incompatible. As already stated, it has been the experience of those working with rabbits that when such evidence of incompatibility fails to appear, transfused blood is well tolerated. In the present instances agglutination was not encountered. As a further check a rabbit was transfused with frankly incompatible blood to learn whether its destruction would lead to a mounting hemoglobin percentage. That in this instance the strange blood was promptly destroyed can be inferred from the fact that the hemoglobin underwent none of the increase seen in the animals receiving compatible blood (Chart 6); yet the reticulocytes remained at the pretransfusion level. Throughout the transfusion period there was a lack of significant intercurrent variations in the number of these cells, such as might have been expected had the marrow been subjected to restimulation.

From all this it seems plain that the marrow activity during the period while the hemoglobin was increasing, must have been due, not to stimulation by the products of blood destruction, but to persistence at a normal task.

The Effects of Repeated Small Blood Losses

The lack of sensitiveness of the marrow to induced increases in hemoglobin contrasts strikingly with its response to slight blood losses. It has been said that in the best controlled experiment, that of the third series of animals, observations were made on the donors at the same time as on the recipients. The loss sustained by these donors did not exceed by as much as a drop per day the amount of blood gained by the recipients. Yet in all six instances (Charts 7, 8, and 9) the percentage of reticulocytes markedly increased within a few days after

the bleedings had been begun, and though the blood losses caused but a transient fall, when any, in the hemoglobin percentage, and were soon followed by recovery to a higher percentage than before, the reticulocyte count persisted above the previous level so long as blood was withdrawn. When no more was taken, the count soon fell, and reached the pretransfusion level. It is plain that not only did the erythropoietic tissue become abnormally active under the stimulus of the bleedings, but it never adjusted itself to the daily loss, being

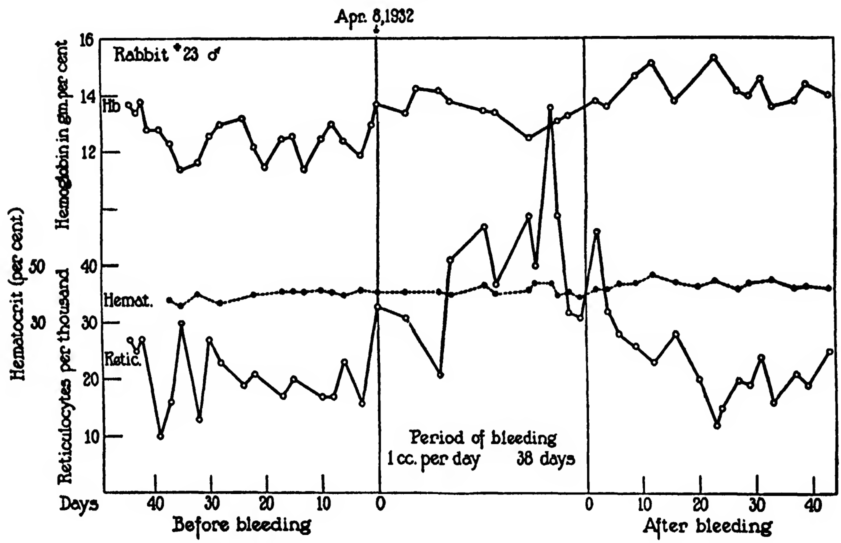

CHART 7

quite as sensitive thereto at the end of the period of hemorrhage as at the beginning.

The hemoglobin percentage of the donor rabbits,—which throughout remained in excellent condition,—continued to rise progressively after the bleedings had been stopped, surpassing the previous normal, but not attaining the level reached in some of the recipient rabbits. This rise may conceivably have been due to overcompensation, such as is frequently observed after hemorrhage, though the effect of this does not ordinarily persist for so long a period.

The observation that daily small blood losses in healthy animals may not only be repaired, but may be attended by an increase in the amount

of circulating hemoglobin, assumes special significance when the consequences are studied of removing the same total amount of blood on fewer occasions. The experiment was carried out on four rabbits selected and studied with the donors of the third experiment but deprived of 7 cc. of blood at a single bleeding once a week. In all four the reticulocyte percentage rose far more markedly than in the animals bled 1 cc. per day, yet the manufacture of blood was insufficient to compensate for the weekly loss and an anemia developed (Charts 10

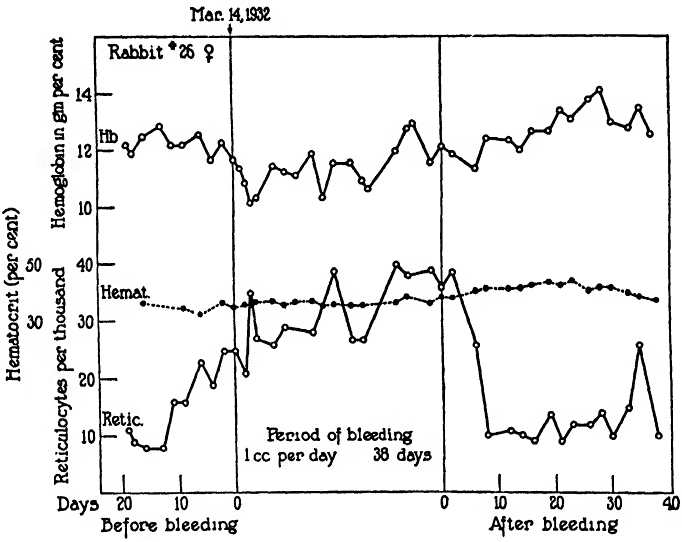

Chart 8

and 11). The chart of the average findings (Chart 11) typifies the individual instances. There was a far greater increase in the number of reticulated cells than when the blood was withdrawn as 1 cc. per day. The blood removed was in one case injected into another, compatible rabbit. The hemoglobin of this animal was increased from 13.0 gm. to 14.2 gm. and a pronounced depression of marrow activity took place as evidenced by the reticulocyte change.

Habituation to Overmuch Hemoglobin

The fact that the erythropoietic tissue is insensitive to gradual additions to the amount of circulating blood pigment, while responding

practically at once to withdrawals of the same magnitude, was evident in the present experiments not only during the period of transfusion, or of bleeding, but in the subsequent weeks, during which the blood studies were continued. For some days after the transfusions of 1 cc. of blood had been stopped the induced superabundance of hemoglobin persisted without change. Then the pigment percentage began to fall; and soon afterwards the erythropoietic tissue became unusually active, —as shown by a sharp rise in the reticulocytes,—and the high percentage was regained. This happened in the rabbits of all three series, but it was best studied, because best controlled, in the rabbits of the

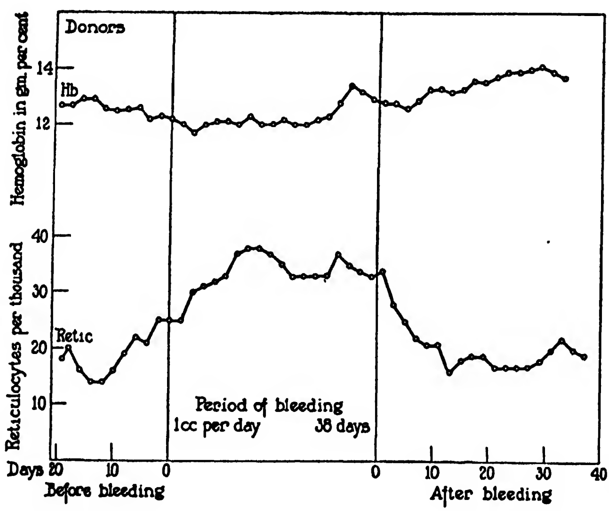

CHART 9. Composite chart of animals deprived of small amounts of blood daily.

third group that received compatible blood (Charts 3, 4, and 5). The phenomenon occurred in all five of these animals. The high hemoglobin level was maintained throughout some weeks of observation, that is to say until expediency rendered it necessary to terminate the experiments; and the induced marrow activity, though not so great as after the hemoglobin first fell off, only gradually diminished to the normal rate as indicated by the percentage of reticulocytes. Evidently the organism had been so altered by the experimental procedures that a much higher hemoglobin percentage than that obtaining prior to the transfusions was now normal to it.

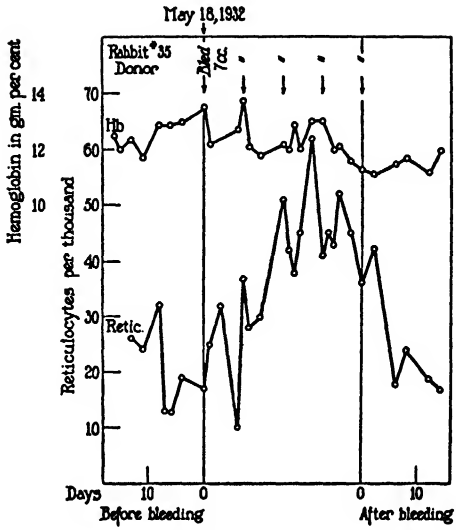

CHART 10

DISCUSSION

The initial object of the experiments was to determine the rate of blood production. It was supposed, on inference from indications here and there in the literature, that this could be done by supplying compatible blood to the normal organism in precisely the amount daily produced by the erythropoietic tissue, whereupon presumably this tissue would cease work. What occurred was wholly different. The day to day introduction into the circulation of small amounts of blood was without perceptible influence on bone marrow activity and the hemoglobin percentage rose gradually yet markedly. Its activity continued unabated throughout the transfusion period and must have contributed in no small part to the rise in hemoglobin. The grounds for these conclusions have been considered in the text.

If, in some of the recipient animals, it is possible to suppose that the increase in hemoglobin represented merely blood betterment over a previous low normal as result of favorable conditions (Charts 1 and 2), in the case of others (Charts 3 and 5) one is forced to look upon the state of affairs brought about by the additions of blood as constituting a superabundance for reasons that have been given. The findings as

concerns marrow activity were similar in all of the recipients of small amounts of blood. They indicated that blood formation was practically unaffected by the gradually mounting hemoglobin (Charts 3, 4, and 5).

As already stated, Boycott and Oakley (2) transfused rabbits repeatedly with large amounts of blood to determine whether, as result of the provision of cells from without, the marrow would not atrophy or at least stop work. They found, as had Robertson before them, that the reticulocytes soon fell to zero; but although the transfusions were kept up this change did not endure but with every inter-

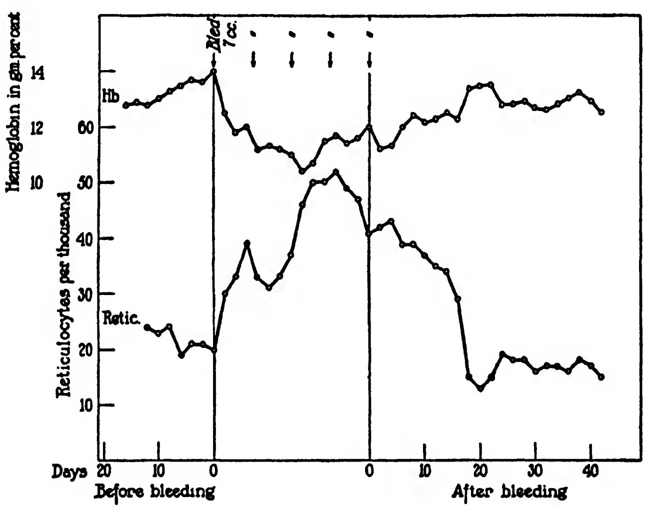

CHART 11. Composite chart of animals bled at weekly intervals.

current fall in hemoglobin from the abnormally high level,—and these were frequent,—the reticulocytes reappeared in greater or less number. Boycott and Oakley did not ensure compatible blood by preliminary tests; and the difficulties they mention of maintaining a superabundance of hemoglobin, difficulties evident in their charts, indicate that now and again the alien corpuscles must have been abruptly destroyed in quantity, a fact which they themselves recognized, doing control experiments on the possible effects of blood destruction to cause marrow stimulation. They could not bring the marrow completely to a standstill, though, as they remark, the abundance of transfused cells might be presumed to have saved the organ from the necessity of making any fresh ones.

Evidence was obtained in all of the recipient groups of the present work that habituation develops to an increased amount of circulating

hemoglobin when this increase is brought about gradually. Soon after the daily transfusions were stopped, the hemoglobin percentage began to decline from its high level, but long before it had progressed far in the direction of the previous normal, the marrow became unusually active, as shown by a pronounced increase in the number of reticulated cells, and the high pigment level was restored and maintained (Charts 3, 4, and 5). The possibility that the increased erythropoietic activity resulted from the stimulation due to intercurrent blood destruction can be dismissed for reasons already given. Unfortunately the animals had to be sacrificed within a few weeks after the transfusions had been stopped, so the late consequences of these remain unknown.

Robertson showed that when a superabundance of hemoglobin had existed for some time the withdrawal of an amount of blood insufficient to bring the amount of pigment to the pretransfusion level resulted nevertheless in an increase in bone marrow activity. In doing this experiment he abruptly and considerably reduced the blood bulk. That considerable blood reductions stimulate the marrow of the normal animal, as repeated small losses to the same total amount do not, is shown by the large increase in the reticulocytes in our rabbits that were bled 7 cc. once a week as compared with the relatively slight increase in those bled 1 cc. *per dicm*; and it might be inferred that Robertson's finding was the result merely of a drastic change in the blood bulk. This possibility was controlled in the transfused rabbits of the present work. Hemorrhage was not the cause of the post-transfusion fall in hemoglobin which resulted in marrow stimulation, nor, for that matter, was the reduction in the pigment percentage a great one.

Subsidiary evidence on the insensitiveness of the marrow to hemoglobin increases when these occur gradually can perhaps be found in the data on the donors for the third group of transfused rabbits. Although these lost 1 cc. of blood per day the hemoglobin percentages had returned to the initial level when the losses were stopped, and soon thereafter they rose above it. Thoughout the period of the bleedings the marrow had been unusually active as shown by the increased percentage of reticulocytes; but when they were discontinued, the number of these cells declined to the previous normal. Below this normal they did not fall despite the fact that the hemoglobin percentage continued to mount.

The conception of habituation to superabundance of hemoglobin would seem on first consideration to be excluded by the numerous observations which attest to the fact that induced alterations in the oxygen supply to the body cause changes in the activity of the erythropoietic tissue. But the contradiction is only an apparent one. True, when an animal is suddenly injected with a large amount of compatible blood, or is abruptly placed in an atmosphere abnormally rich in oxygen, its marrow slows in the production of red cells. Any abrupt and sustained large increase or decrease in the opportunity for oxygen evokes an alteration in the rate of erythropoiesis. But it does not *follow that such an alteration will be elicited when the change is small or gradual. Interpolated between the supply of oxygen and the needs* of the tissues are a number of physiological mechanisms, circulatory and respiratory in the main, which act to minimize or ward off completely the stimulus to the marrow of incidental changes in oxygen demand or supply. Were it not for the working of these mechanisms the erythropoietic system would be having to respond to constantly recurring buffets in the form of demands, quickening its activity after an individual had played tennis, slackening when he had spent a day or so in bed. But because of the protection afforded by intermediate mechanisms, transient changes in oxygen demand or supply, those normal to ordinary life, must scarcely be felt as stimuli by the erythropoietic tissue, if felt at all.

Boycott and Oakley draw attention to some of the resources of the organism, other than erythropoiesis, that are utilized in coping with changes in oxygen supply or demand and they provide an unique example in which all such resources were overborne. Rabbits were transfused excessively, with result in so great an increase in the viscosity of the blood that it could not be circulated well enough to prevent anoxemia of the tissues. Despite the immense quantity of hemoglobin present in circulation the erythropoietic tissue became unusually active, just as if there were a pigment deficiency.

Granting that the physiological mechanisms mediating between the available oxygen supply and the needs of the tissues suffice to protect the red marrow from having to respond to transient emergencies, is it possible that gradual changes in oxygen supply or demand are ever coped with over a long period by these mechanisms alone? The literature does not provide a comprehensive answer to this question.

Observations on the effects of changes in altitude upon the blood have in general been conducted upon persons whose environment in such respect has been changed suddenly; while animals studied with relation to the effects of changes in the amount of oxygen in the inspired air have as a rule been suddenly placed in atmospheres poor or rich in this gas and as abruptly removed therefrom. There can be no doubt that a prolonged sojourn in rarefied air is attended by an increase in circulating hemoglobin and that persons living all their lives at high altitudes have in general a greater hemoglobin percentage than natives of the lowlands. In most instances the amount of the pigment varies directly with the availability of oxygen. That this is not always the case is indicated by the observations of Somervell upon two Tibetans who had lived most of their lives at a height of 16,500 feet. Their hemoglobin percentages were only 92 and 82 respectively, yet they could race up steep slopes "twice as fast" as the English climbers whose hemoglobin percentages had mounted to an average of 120 as result of the change in altitude. The average hemoglobin quantity in Hurtado's large series of individuals living at 14,900 feet was only 15.93 gm. per cent, as compared with 15.75 gm. per cent for those at sea level whom he studied. Campbell found the oxygen tension in the tissues of a number of normal rabbits to be identical despite wide individual variations in the hemoglobin percentage,—though abrupt, great changes in this percentage, brought about by experimental means, resulted in changes in the tension.

These various findings indicate that the organism does not necessarily make more blood or less blood when the opportunity for oxygen is large or small throughout a protracted period, but may on occasion have recourse for adjustment to its other resources.

There are many clinical reports of obdurate anemia resulting from repeated slight hemorrhages which might be taken to indicate that the organism can become habituated to a smaller amount of circulating hemoglobin than the normal, just as it becomes habituated to a larger one, according to the findings here reported. Campbell's observations, above referred to, on the identical oxygen tension of the tissues in normal rabbits with widely differing hemoglobin percentages provide good evidence of habituation to a relative anemia. Yet in the present experiments the organism did not adjust itself to repeated small hemorrhages to such extent that the erythropoietic tissue failed to be stimulated by them. At the end of many daily losses of 1 cc. of blood,—about 1/100 of the total quantity,—it was still responding as actively as at first, and this although the hemoglobin percentage had returned to the initial level. Very possibly the marrow was re-

sponding to the repeated slight diminutions in blood bulk as such. The great response to bleeding 7 cc. once a week, as compared with 1 cc. per day, and the differing course of the hemoglobin curves, illustrate a point which deserves reiteration; namely, that the consequences of considerable and abrupt changes in blood volume and hemoglobin percentage provide no sufficient basis upon which to predict the outcome of repeated small ones to the same total amount.

The facts do not enable one to say whether the rabbits manifesting habituation to an increased amount of hemoglobin would have continued to keep this amount in circulation for a long period. Plainly the change in their circumstances had greatly altered their case. But whatever this case the hemoglobin percentage maintained by them would, in the absence of abnormal blood destruction, necessarily have been, in last analysis, the resultant of the forces making for depression and stimulation of the marrow respectively, just as in normal animals. According to Boycott and Oakley "The normal animal is evidently working about a nice level of delicate balance, which is presumably the reason for the constant presence of a few reticulated cells." A balance there certainly is, but hardly a nice one. For the present work has disclosed the fact that the erythropoietic tissue is insensitive to the effects of repeated, small blood accretions, though very sensitive to blood losses of the same magnitude. This is what one would expect if, throughout the course of age-long differentiation and selective survival, body needs have had the effect of determining body capabilities. The normal organism has always had to cope with accidental losses of hemoglobin, if it was to survive, but almost never with a superabundance of the pigment.

SUMMARY

The effects of very gradually increasing or diminishing the amount of circulating hemoglobin have been studied in rabbits. Contrary to expectation it was found that when the pigment was increased by the injection of a small quantity of compatible blood every day during some weeks the erythropoietic tissue did not lessen its activities. The hemoglobin percentage mounted gradually yet considerably when even as little as 1/100 of the amount of blood initially possessed by the animal was injected each day; and the figure it finally attained must in

some instances at least have been expressive of a superabundance. To this superabundance the animal itself evidently contributed through its persisting erythropoietic activity.

The results were very different when rabbits were bled daily to the same small amount that was injected into their fellows. The marrow became abnormally active, and this activity continued undiminished throughout the long period of the bleedings. The organism is evidently far more susceptible to blood losses than to blood gains, a fact which is scarcely surprising when one considers that throughout its differentiation as a going concern it has had to cope with exigencies of the first sort only.

Rabbits in which the hemoglobin is very gradually increased by the injection of strange blood become so accustomed to the abundance of pigment that even a slight falling off causes the erythropoietic tissue to become abnormally active to maintain the new *status quo*. Good reasons exist for referring the habituation thus manifested to readjustments in the functioning of the physiological mechanisms which mediate between oxygen demand and erythropoietic response. Too little recognition has been given to the rôle of these mechanisms in such relation. No evidence was obtained of any effective readjustment to protect the erythropoietic tissue from the stimulus of daily small blood losses.

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REACTION OF TRANSPLANTABLE AND SPONTANEOUS TUMORS TO BLOOD-CARRIED BACTERIAL TOXINS IN ANIMALS UNSUSCEPTIBLE TO THE SHWARTZMAN PHENOMENON

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Gratia and Linz[1] described a phenomenon characterized by a violent hemorrhagic and necrotic reaction in the tumor in guinea pigs bearing large transplantable lipo-sarcomas, shortly after intravenous injection of a filtrate of *B. coli* broth culture. The authors identified this phenomenon with that of "Sanarelli-Shwartzman", and also with similar focal hemorrhagic reactions resulting from intravenous injection of bacterial filtrates in rabbits injected with a number of bacteria and filterable viruses.* Shwartzman and Michalowsky[3] described the same phenomenon in a rather extensive series of mice bearing Sarcoma 180. Guinea pigs as well as mice may die within 24 hours following the injection.[1,3] Some of the mouse tumors may temporarily regress or completely disappear.

Besides their intrinsic value, 2 considerations make these findings particularly interesting: (1) mice and rats are unsusceptible to the ordinary Shwartzman phenomenon, and it would seem that special conditions existing in the tumor render its vessels, supplied by the host, apt to react with the blood-carried bacterial toxins. (2) This state of reactivity of the tumor in either susceptible or non-susceptible animals is a permanent one, without the need of any previous local injection of bacterial filtrate as is the case with the ordinary Shwartzman phenomenon in rabbits and guinea pigs.

[1] Gratia, A., and Linz, R., *Compt. rend. Soc. biol.*, 1931, **108**, 427; *Ann. Inst. Pasteur*, 1932, **49**, 131.

* As early as 1907 S. P. Beebe and M. Tracy[2] described the regression of several cases of dog lymphosarcoma under the influence of the "Coley toxin" from *Streptococcus*, *B. coli*, and *B. prodigiosus*.

[2] Beebe, S. P., and Tracy, M., *J. Am. Med. Assn.*, 1907, **49**, 1493.

[3] Shwartzman, G., and Michalowsky, N., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 737.

We have studied the phenomenon described by Gratia and Linz on a variety of neoplastic processes in animals unsusceptible to the Shwartzman phenomenon, namely, rats and mice. As a source of bacterial toxin we used filtrates of 6-day-old broth cultures of *B. coli* and injected them into the peritoneum or vein in the amount of 0.5 cc. for mice and 1 cc. for rats. We have divided our results into 2 groups, according to whether the phenomenon was positive or negative.

1st group (*positively reacting tumors*). This group includes 203 mice or rats bearing rapidly growing malignant transplantable tumors which give a high percentage of takes and rarely retrogress. The tumors differed widely in age, size, and degree of necrosis at the time of the toxin injection. The results are summarized in Table I.

TABLE I

No. of animals	Tumor	% showing positive phenomenon	% of regressed tumors	% death within 24 hours following injection
61 mice	S/37 sarcoma	67	20	0
68 "	180 "	60	10	10
21 "	M/63 adeno-carcinoma	57	0	47
19 "	Twort " "	70	0	0
34 rats	Walker sarcoma	88	0	41

In the tumors included in Table I the intensity of the phenomenon bears a direct relationship with the age and size of the tumors. While very young, perfectly healthy tumors often give a negative phenomenon, large tumors, averaging 1x1 cm. for mice and 2x1 cm. for rats, give practically 100% positive results. The strongly positive phenomenon is characterized by an extensive hemorrhagic condition, which in most cases may be observed externally through the skin. A more moderate phenomenon requires the opening of the growth to be detected. The very necrotic parts, where vessels are obliterated, are naturally free from hemorrhage. In the more prominent cases the phenomenon can be detected in the tumor as early as one hour after the peritoneal injection of bacterial filtrate.

2nd group (*negatively reacting tumors*). This group includes 69 mice and rats bearing: (1) comparatively slowly growing spontaneous or transplantable malignant tumors which rarely or never regress,

(2) rapidly growing malignant tumors which eventually regress (heterologous graft), and (3) benign embryomas or granulomas, rapidly developing, which eventually regress. The detailed account is as follows:

19 mice bearing spontaneous malignant mammary adeno-carcinomas.

6 mice bearing the transplantable malignant Harding and Passey melanotic sarcoma.

4 mice bearing the transplantable malignant Walker rat tumor.

10 rats bearing the transplantable malignant S/37 or 180 mouse sarcoma.

20 mice bearing benign embryomas.

10 mice bearing kieselguhr granulomas.

None of these tumors or granulomas showed any definite hemorrhagic phenomenon. Only 2 spontaneous tumors and 2 mouse tumors growing in rats showed a doubtful mild phenomenon. Three spontaneous tumors were tested a second time 24 hours after the tumor had been "prepared" by injection of 0.2 cc. of filtrate into the mass. Again no modification in the tumor could be noticed. It is worth pointing out that some of the mouse tumors growing in rats had at the time of injection attained a mass of 2.2x0.9 cm. of perfectly healthy tissue with actively dividing cells and apparently perfect blood supply, as shown by histological examination. Such growths were the result of grafting large pieces of tumors under the skin of the heterologous host.

From the results obtained with the tumors so far studied, it appears that only those growths showing at the same time *malignancy* and *rapidity of growth* show the phenomenon of Gratia and Linz.

The mechanism of death as a result of the reaction in the tumor is not understood, and no significant general lesions have been found at the autopsies. Numerous control tests have shown that normal mice and rats stand the injection of the bacterial filtrate without marked alterations in their health. Additional experiments have shown that the general resistance of the animals to the ordinary Shwartzman phenomenon is not changed by the existence of the tumor. Also small tumors may be found unsusceptible despite their growing in an animal bearing large susceptible tumors. The fre-

quency of death seems to be in a direct relationship with the size and age of the tumor, but besides this there is another intrinsic factor depending on the strain of tumor itself. The Bashford adeno-carcinoma seems, from this point of view, to be the most "toxic" of all the strains so far studied.

Animals in which, as a consequence of the phenomenon, the tumors regressed were found resistant to regrafting. The intimate cause of this tumor regression is now being investigated.

In view of the fact that transplantable tumors are often infected by various bacteria, we have secured sterile tumors, and found that they are as sensitive to the phenomenon as the infected ones. Moreover, filtrates of tumors showing a strong phenomenon, injected into the skin of rabbits, have failed to sensitize this organ to intravenous injection of a potent bacterial filtrate made 24 hours later.

Work is underway extending these studies to more true tumors and granulomas occurring in animals both susceptible and resistant to the ordinary Shwartzman phenomenon in order to obtain more information about the relationship existing between malignancy and rapidity of growth on the one hand, and ability to react to bacterial toxins on the other hand.

Summary. Whereas rapidly growing transplantable malignant tumors in rats and mice are very susceptible to blood-carried *B. coli* toxin, slow-growing malignant spontaneous or transplantable tumors, malignant tumors rapidly growing in heterologous hosts, embryomas, and granulomas are practically non-susceptible.

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THE BLOOD CYTOLOGY IN UNTREATED AND TREATED SYPHILIS

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Improved methods for the study and differentiation of living cells based on physiologic activity have provided means for investigating blood and tissue reactions from a functional as well as a morphologic point of view. These methods have been applied to the study of syphilitic reactions in man[1] and animals.[2] The present report is concerned with the blood cytology, as determined by the supravital technique, of patients with untreated and with treated syphilis. A subsequent paper will discuss the blood cytology in the active and latent stages of the experimental disease.

MATERIAL AND METHODS. Observations were made on the blood cytology of 213 syphilitic patients.* Of these, 87 had received no treatment, and 126 had been variously treated before the blood examinations were made. The untreated group was composed of 48 patients with active primary infection, 23 patients who had active secondary lesions, and 16 who presented signs and symptoms of tertiary disease. The treated group consisted of 43 patients on whom treatment was started in the primary stage, 30 who had received their first treatment in the secondary stage, and 53 who began their treatment in the tertiary phase. The clinical diagnosis of syphilis was confirmed in all cases by dark-field or serologic examination. This report is essentially limited to the study of white males. Of the 213 patients, 29 were colored and 7 were females.

The blood examinations were conducted from November, 1930, to November, 1932; with the exception of March, 1931, and July, August, and September of 1931 and 1932, counts were made every month during this interval. Since the patients were ambulatory and repeated

* Through the kindness of Dr. Howard Fox, these patients were made available for study in the Syphilis Clinic of the University and Bellevue Hospital Medical College, New York University.

counts could not readily be obtained, only one complete blood examination was made on each individual. Each examination included a total white and red cell count made with standardized automatic pipettes, a platelet count by the Ringer-heparin method of Casey,[3] a hemoglobin determination by the Newcomer method, and a differential white cell count, using the supravital neutral red technique, 100 cells being counted on each of two smears. The blood samples were taken in the afternoon, usually between 2.00 and 3.00 P.M., and examined later[4] the same afternoon. From 2 to 7 blood examinations were made on those afternoons devoted to this study. One observer made all the red cell, white cell, platelet, and hemoglobin determinations, and all the differential smears were examined by the same two observers.

Most of the treated patients received alternating doses of neoarsphenamin and mercury, but many had been treated either entirely or at some time with various drugs, including bismuth, arsphenamin, sulpharsphenamin, silver arsphenamin, and tryparsamid. The amount of treatment ranged from less than one course to several courses separated by rest periods. Classification on the basis of the drugs used, the dose, and frequency of administration has not been attempted.

In the analysis of the results, the usual statistical methods were employed for determining the mean, the standard error of the mean, and the standard error of the difference of two means. A difference was considered significant when the probability of its occurrence by chance was less than one in 100 ($t = 2.5$, $P = 0.01$). The χ^2 method is that described by Fischer.[5]

It is usual in a study of the blood picture in any disease to indicate whether the findings are higher, lower, or the equivalent of normal values. In the present study there were two objections to this procedure. The normal limits of variation in the number of white blood cells in man is usually given as ranging between 5000 and 10,000, with an average of 7500. In our experience of weekly counts on a small group of normal young men over a period of 2 years,[6] the mean total white cell count was nearer 6500 than 7500. In addition to this discrepancy between accepted values and our own for the total white cell count, a second difficulty was the fact that, so far as is known, there has not yet appeared an adequate statistical survey of

the differential cell formula of normal individuals as determined by the neutral red supravital technique, the method employed in this study. Because of these two facts, we have hesitated to ascribe normality to any of the values obtained in this investigation.

We have, however, made an effort to determine the differences, if any existed, between the supravital and fixed smear methods in our hands. Duplicate counts were made on the first 86 patients of this series with the two techniques. The cover-slip method was employed in the preparation of the fixed smears, and 100 cells were counted on each of two smears after staining by Wright's method. A comparison of the mean values for the counts on these 86 individuals revealed that significant differences were present. Counts made with the supravital technique gave significantly higher values for neutrophils, basophils, and monocytes, and a significantly lower value for lymphocytes, than did duplicate counts on fixed smears. The eosinophils were slightly higher with the neutral red method, but this difference was not significant. (Neutrophils: Difference, 2.4 ± 0.97 per cent; t, 2.5; P, 0.01. Basophils: Difference, 0.3 ± 0.10 per cent; t, 3.0; P, 0.01. Monocytes: Difference, 1.7 ± 0.53 per cent; t, 3.2; P, 0.01. Lymphocytes: Difference, 4.7 ± 0.89 per cent; t, 5.3; P, 0.01. Eosinophils: Difference, 0.3 ± 0.29 per cent; t, 1.1; P, 0.2.) With the exception of the findings for monocytes, the direction of these differences was similar to that observed by Sabin.[7]

Since comparisons with normal values could not be made, the procedure adopted was to group the patients in a systematic manner, and compare the blood cell values of one group with those of another. In this way errors which might be due to variations in technique, in observers, and countries are minimized.

RESULTS. The essential findings are summarized in Tables 1 to 7. The values given in Tables 1 and 2 are represented graphically in Figure 1.

The Blood Cytology in Untreated Syphilis. The mean blood cell values of the untreated patients whose blood was examined in the primary, secondary, or tertiary stages are shown in Table 1. No significant differences were found between any of the blood elements of the primary and secondary cases. The comparative infrequency of untreated tertiary syphilis is evidenced by the small number of individ-

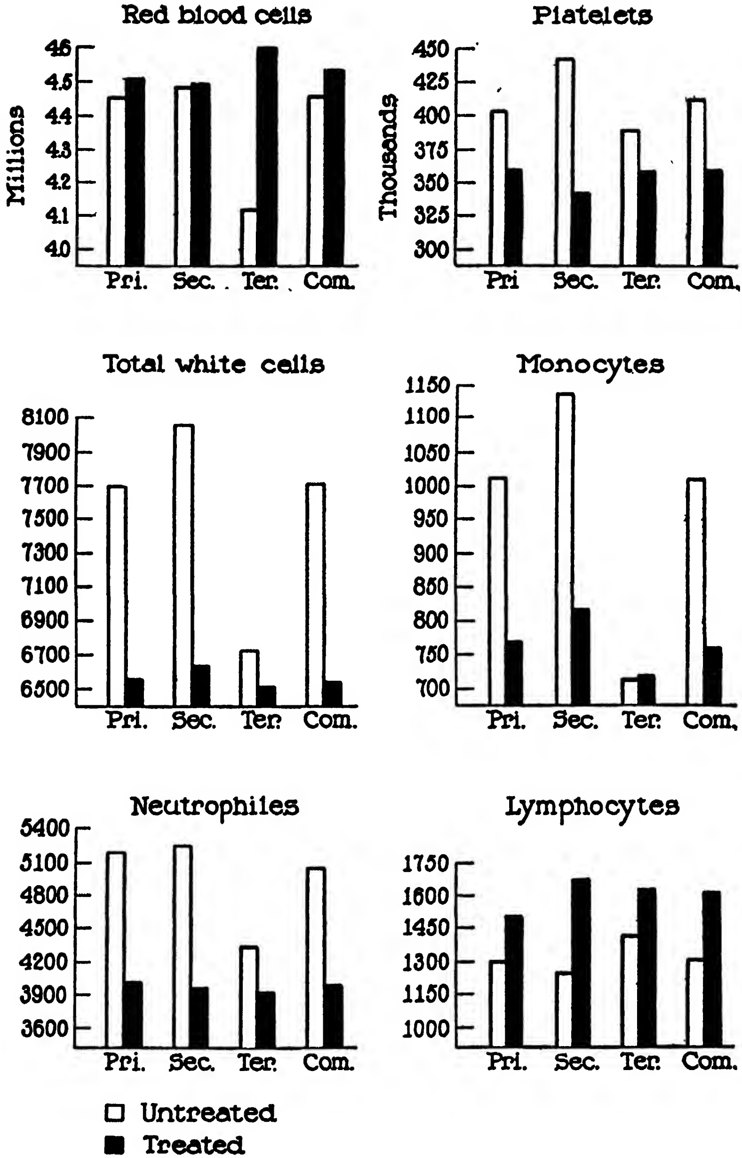

FIG. 1.—The blood cytology in untreated and treated syphilis.

Pri., Sec., and Ter. refer to the primary, secondary, and tertiary stage. Com. indicates the combined values for all three stages.

TABLE 1

The Mean and Standard Error of the Mean of the Blood Constituents in Untreated Syphilis

Stage.	No.	Value.	R. B. C.	H.	P.	W. B. C.	N.	B.	E.	L.	M.
Primary	48	Mean	+000 4456	% 69.0	+000 403	7696	5187	37	155	1306	1010
		Standard error of mean	±77.1	±1.3	±5 2	±278 8	±233 6	±8.3	±21.0	±40.7	±59.9
Secondary	23	Mean	4477	63.8	441	8052	5247	63	323	1277	1137
		Standard error of mean	±123.5	±1.8	±25.7	±529.1	±377.5	±13.4	±82.8	±113.6	±86.7
Tertiary	16	Mean	4119	65.5	389	6716	4355	62	184	1409	712
		Standard error of mean	±122.5	±1.7	±22.3	±442.9	±365.4	±11.8	±36.7	±115.9	±76.8
Total	87	Mean	4455	67.0	411	7610	5050	48	205	1316	989
		Standard error of mean	±60.7	±1.0	±9.4	±228.7	±179.9	±6 5	±26.7	±51.0	±45.0

R. B. C., red blood cell count; W. B. C., white blood cell count; P., platelet count; H., hemoglobin; N., neutrophils; B., basophils; E., eosinophils; L., lymphocytes; M., monocytes.

TABLE 2

The Mean and Standard Error of the Mean of the Blood Constituents in Treated Syphilis

Stage.	No.	Value.	R. B. C.	H.	P.	W. B. C.	N.	B.	E.	L.	M.
Primary	43	Mean	+000 4511	% 72.5	+000 358	6563	4039	59	192	1502	771
		Standard error of mean	±90.0	±1.1	±11.5	±238.2	±179.9	±9.8	±29.2	±74.9	±39.2
Secondary	30	Mean	4487	72.9	341	6632	3941	46	147	1677	821
		Standard error of mean	±104.9	±1.9	±18.3	±318.6	±228.3	±9.8	±23.0	±124.4	±52.7
Tertiary	53	Mean	4599	71.6	358	6511	3921	50	172	1650	718
		Standard error of mean	±104.9	±1.5	±9.9	±254.9	±171.2	±13.5	±17.3	±59.5	±37.0
Total	126	Mean	4542	72.3	358	6559	3966	52	174	1606	761
		Standard error of mean	±53.8	±0.8	±6.2	±153.2	±107.6	±6.2	±13.3	±46.5	±24.1

R. B. C., red blood cell count; W. B. C., white blood cell count; P., platelet count; H., hemoglobin; N., neutophils; B., basophils; E., eosinophils; L., lymphocytes; M., monocytes. The designations, *primary*, *secondary*, and *tertiary*, refer to the stage in which treatment was begun.

uals in this group. There were a sufficient number of observations, however, to show that the mean monocyte count was significantly lower than that for both the primary and secondary cases. (Primary: Difference, 297.8 ± 97.3; t, 3.06; P, 0.01. Secondary: Difference, 425.3 ± 115.7; t, 3.67; P, 0.01.) Of interest also is the low mean value for the red cell count in the tertiary group. This was significantly lower than the red cell count in the primary syphilitics, and probably significantly lower than the mean red cell count of the patients with secondary disease. (Primary: Difference, 437,000 ± 14,500; t, 3.01; P, 0.1. Secondary: Difference, 358,000 ± 17,300; t, 2.06; P, 0.05.) It will be noted that although the differences were not statistically significant, the values for the total white cell count, platelet count, and neutrophils were lower in tertiary syphilis, while the lymphocytes were higher than in either of the early stages. These differences no doubt would have been of greater significance had the number of patients in the tertiary group been larger. A comparison of the findings in late syphilis (tertiary stage) with the combined values for early syphilis (primary and secondary stages), gave two highly significant differences. In the late group of cases both the red cell count and the monocyte count were significantly lower than the corresponding values for the early syphilis group. (Red cells—combined primary and secondary group: n, 71; tertiary group: n^1, 16. $Mean_n$, 4,530,600 ± 6,580. $Mean_n$, 4,119,000 ± 12,240. Difference, 411,600 ± 13,900; t, 2.96; P, 0.01. Monocytes—$Mean_n$, 1,051 ± 49. $Mean_n$, 712 ± 77. Difference, 339 ± 91; t, 3.71; P, 0.01.) On the same basis none of the other blood elements showed any statistically significant differences.

The Blood Cytology in Treated Syphilis (Table 2). Three subdivisions were employed in the analysis of these results, depending on whether treatment was begun in the primary, secondary, or tertiary stage of the disease. No significant differences were observed in the blood formulae of the three groups.

Comparison of the Results for Treated and Untreated Syphilis. The differences in the untreated and treated groups (Tables 1 and 2) were submitted to a further analysis. The mean values for the combined untreated group of 87 patients were compared with those of the combined treated group of 126 patients. In the treated group, the total

white cell count was lower, the platelet count was lower, the hemoglobin in per cent was higher, the absolute number of lymphocytes was higher, and the absolute number of neutrophils and of monocytes was lower, than the corresponding values for the untreated group. (Total white cells—Difference, 1,051 ± 275; t, 3.8; P, 0.01. Platelets—Difference, 53,000 ± 11,000; t, 4.7; P, 0.01. Hemoglobin—Difference, 5.3 ± 1.10 per cent; t, 3.6; P, 0.01. Neutrophils—Difference, 1,084 ± 210; t, 5.2; P, 0.01. Lymphocytes—Difference, 290 ± 69; t, 4.2; P, 0.01. Monocytes—Difference, 228 ± 51; t, 4.5; P, 0.01.) No difference was observed between the two groups with respect to the red cell count or the absolute numbers of eosinophils and basophils.

In relative per cent the same differences were noted between the white cell formulæ of the treated and untreated groups as for the absolute numbers of the white cell components (Table 3). It will be seen that the neutrophils and monocytes of the treated group were lower, and the lymphocytes higher, than the corresponding cells in the untreated group.

Discussion. Thus the mean cell values of the total treated group of 126 patients differed from those of the untreated group of 87 patients in the following respects: Lower total white cell count, platelet, neutrophil, and monocyte counts, and higher values for lymphocytes and hemoglobin. There are certain factors in the material itself which might have influenced these differences. These will be discussed in order.

Number of Observations. One factor which might account for the observed differences is the disproportion between the numbers of patients in the treated and untreated groups. The untreated series had only 16 patients in the tertiary group, while the treated series contained 53 patients who received their first specific treatment in the tertiary stage (Tables 1 and 2). Could this bias account for the differences between the blood cells of treated and untreated cases? If tertiary cases are omitted from both groups, and a comparison is made between the 71 untreated patients with early syphilis (primary and secondary stages), and the 73 patients treated in the early period, the two groups are more nearly comparable as far as numbers are concerned than if the total treated and untreated groups are considered. But the same differences are apparent as were observed

TABLE 3

Mean Blood Cell Values in Per Cent in 87 Cases of Untreated and 126 Cases of Treated Syphilis

	Neutrophils.		Basophils.		Eosinophils.		Lymphocytes.		Monocytes.	
	Untr.	Tr.	Untr.	Tr.	Untr.	Tr.	Untr.	Tr.	Untr.	Tr.
	%	%	%	%	%	%	%	%	%	%
Mean	65.6	59.8	0.6	0.7	2.7	2.6	17.9	24.9	13.1	11.7
Standard error of mean	±0.84	±0 77	±0.02	±0 02	±0.25	±0.26	±0.65	±0.64	±0.45	±0.32
Difference between means	5.8 ± 1.13		0.1 ± 0.11		0.1 ± 0.36		7.0 ± 0.91		1.4 ± 0.55	
t	5.2		0.9		0.3		7.7		2.6	
P	0.01		0 3		0.8		0 01		0.01	

Untr. = Untreated. Tr. = Treated.

TABLE 4

Mean Blood Cell Values in 73 Cases of Untreated Syphilis and 71 Cases of Treated Syphilis. Combined Values for Primary and Secondary Stage

	Platelets.		Hemoglobin.		White blood cells.		Neutrophils.		Lymphocytes.		Monocytes.	
	Untr.	Tr.	Untr.	Tr.	Untr	Tr.	Untr.	Tr.	Untr.	Tr.	Untr.	Tr.
	+000	+000	%	%								
Mean	416	351	67.3	72.7	7812	6577	5206	3999	1296	1574	1051	792
Standard error of mean	±9 8	±10.1	±1.18	±0.69	±255.9	±190.6	±200.0	±138.9	±94.4	±67.8	±49.4	±31.6
Difference between means	65 ± 14.0		5.4 ± 1.37		1235 ± 319		1207 ± 243.4		278 ± 116.2		259 ± 58.7	
t	4.6		3.9		3.9		4.9		2.4		4.4	
P	0.01		0.01		0.01		0.01		0.015		0.01	

Untr. = Untreated. Tr. = treated.

between the total treated and untreated groups (Table 4). The total white count, the platelet count, the neutrophil and monocyte counts in the 73 treated patients were lower, and the lymphocyte count and the hemoglobin in per cent were higher, than the same values for the 71 untreated patients. There was no difference between the red cell values or the numbers of basophils and eosinophils in the two groups. It is evident, therefore, that the disproportion in the number of observations in the two groups did not introduce sufficient bias to account for the differences.

Age. The observed differences were not associated with any differences in age. The mean age of the 87 untreated patients was 32.7 $\pm$ 1.37 years, while the mean age of the 126 treated patients was 36.2 $\pm$ 0.94 years. The difference of 3.5 $\pm$ 1.66 is not of statistical significance since it is only 2.1 times the standard error of the difference.

Sex, Color, and Time of Counting. Of the 213 patients examined, 96.7 per cent were males, and 86.4 per cent were white. A bias which might be introduced by sex and color is therefore not effective in the present series. Since all counts were made in the afternoon, time of the day is not a factor which can account for the differences.

Duration of Infection. It has been shown that the values for the total white cell count, platelets, neutrophils, and monocytes of the untreated late syphilis group were lower, and the lymphocytes higher, than the corresponding values for untreated early syphilis. These differences were similar in direction and affected the same cells as the differences noted between the untreated and treated early syphilis groups. Thus the values for untreated late syphilis and those for treated early syphilis differed in the same manner from the values for the untreated patients with early syphilis. It is evident therefore that the duration of the infection is a factor which affects changes in the blood cytology independent of treatment, since many years may elapse between the primary and secondary stages of infection and the occurrence of tertiary disease. On the same basis it is possible that the changes observed in the treated group as compared with the untreated group might have been influenced by spontaneous variations occurring during the period required for treatment, and that these differences might have developed regardless of the institution of treatment. With this possibility in mind, a further analysis of the results was made.

Of the patients whose first treatment was begun in the primary stage, all cases were selected in which from 1 to 6 months had elapsed between the time of the first evidence of infection and the time when the blood cytology was studied. This group of 19 treated primary cases was compared with the 47 untreated cases of primary infection. The same differences were observed between the mean blood cell values of these two groups as were found between the combined treated and untreated groups.

It is evident, therefore, that the observed differences between the treated and untreated groups were associated directly with the institution of anti-syphilitic measures. It is of interest to recall the somewhat similar observations of Hazen.[8] He found that, under treatment, either with mercury or salvarsan or a combination of the two drugs, cases of syphilis show a fall in the total white count and percentage of neutrophils, and a rise in the percentage of lymphocytes. Moreover, the administration of mercury to a group of non-syphilitics caused a slight fall in the total white count and in the relative neutrophil count, and rise in the relative lymphocyte count. A fall in the number of platelets after treatment has been observed by Mu,[9] while Cummer[10] and Eason[11] and others have commented on the anemia of tertiary syphilis.

The Monocyte and Lymphocyte in Syphilis. The monocyte-lymphocyte ratio, which has been suggested by Cunningham, Sabin, and their coworkers as a prognostic aid in tuberculosis, is of especial interest in the present observations. The mean M/L index for the total untreated group of this study was 0.80, while the total treated group gave a significantly lower M/L index of 0.52. This difference was due to a significantly higher lymphocyte and a significantly lower monocyte level in the treated group. The occurrence of an M/L index of less than 0.55 in the treated group, or of more than 0.55 in the untreated group, was significantly more frequent than would be expected from random association ($\chi^2 = 29.51$; $n = 1$, $P = 0.01$). Not only were differences noted between the M/L indices of the untreated and treated groups, but within the treated group also such differences were observed. Among the treated patients there were 20 individuals whose Wassermann was repeatedly negative after intensive treatment. The M/L index of this group was 0.34. The average

M/L index of a group of 25 patients whose Wassermann reaction was positive on more than one occasion after prolonged treatment was 0.60. Thus there appeared to be a relationship between the numbers of lymphocytes and monocytes in the blood stream and activity of the disease on the one hand, and the efficacy of treatment as evidenced by the Wassermann reaction on the other. This relationship was investigated more precisely.

The group of 20 persistently treated patients with negative Wassermann reactions, and the group of 25 persistently treated patients with positive Wassermann reactions were utilized for the analysis. These groups were classified in two ways: as Wassermann positive or negative, and as having an M/L index of less than 0.45, or 0.45 and above

TABLE 5

Intensively Treated Syphilis Cases Classified as to Blood Serology and M/L Ratio. Observed and Expected Values

	Observed			Expected		
	Less than 0.45.	0.45 or more.	Total.	Less than 0.45.	0.45 or more.	Total.
Wassermann positive	4	21	25	10	15	25
Wassermann negative	14	6	20	8	12	20
Total	18	27	45	18	27	45

(Table 5). The observed deviations from expectation were clearly significant ($\chi^2 = 13.50$; $n = 1$, $P = 0.01$). In other words there was a high positive correlation between Wassermann negativity and an M/L index lower than 0.45, and between Wassermann positivity and an M/L index higher than 0.45.

In a similar manner the lymphocytes and monocytes of the two groups were studied. Again the two groups of 20 and 25 treated patients were classified in two ways: as Wassermann positive or negative, and as having a total lymphocyte count of less than 1350 or more than 1350 per cc. (Table 6). The observed findings were significantly independent of expectation ($\chi^2 = 8.10$; $n = 1$, $P = 0.01$). Put in another form, lymphocyte values higher than 1350 were more

frequently associated with negative serology, and lymphocyte values under 1350 were more often associated with positive serology than could be expected from chance association.

The persistently treated group was again divided in two ways: as Wassermann positive or negative, and as having monocyte values of less than 700 or more than 700 per cc. The observed and expected

TABLE 6

Intensively Treated Syphilis Cases Classified As to Blood Serology and Lymphocytes Per Cc. Observed and Expected Values

	Observed			Expected		
	Less than 1350.	More than 1350.	Total.	Less than 1350.	More than 1350.	Total.
Wassermann positive	14	11	25	9.4	15.6	25
Wassermann negative	3	17	20	7.6	12.4	20
Total	17	28	45	17.0	28.0	45

TABLE 7

Intensively Treated Syphilis Cases Classified As to Blood Serology and Monocytes Per Cc. Observed and Expected Values

	Observed			Expected		
	Less than 700.	More than 700.	Total.	Less than 700.	More than 700.	Total.
Wassermann positive	7	18	25	11.7	13.3	25
Wassermann negative	14	6	20	9.3	10.7	20
Total	21	24	45	21.0	24.0	45

values are presented in Table 7. The association of negative serology with monocytes less than 700, and of positive serology with monocytes more than 700 per cc. was significantly more frequent than would be expected from random association ($\chi^2 = 7.98$; $n = 1$, $P = 0.01$).

Comparison of the Blood Cytology in Human and Experimental Syphilis. A study has been made of the blood cytology in the active and

healed phases of experimental syphilis. It was found that certain differences existed between the mean blood cell values of rabbits with active syphilitic lesions and the values for normal control rabbits. In the syphilitic group the total white blood cells, the number of platelets, and the neutrophils and monocytes were significantly higher, while the lymphocytes were significantly lower than the corresponding values for normal animals. In the spontaneously healed or latent phase of the disease, when the rabbits presented no clinically demonstrable evidence of infection, the blood cell values were not different from normal findings with two exceptions: the red blood cell count and the hemoglobin in per cent were lower.

These findings are of interest since they parallel so closely the observations on the human disease reported in this paper. In the human disease as has been stated, treatment causes a fall in the white blood cell count, platelet, neutrophil, and monocyte counts, and a rise in the value for lymphocytes, from the findings in untreated patients. In the experimental disease, spontaneous regression without treatment is accompanied similarly by a fall in the white blood cell count, platelet count, neutrophil and monocyte counts, and a rise in the lymphocyte count.

Another parallelism, between the red cells and hemoglobin of the human and experimental disease, is also striking. With the exception of the red cells, all the blood elements of the untreated tertiary group showed the same changes from the findings in the untreated early syphilis group as were noted as a result of treatment. The marked depression of the red cells would seem to indicate that the prolonged infection affects them more severely than the other blood constituents. Similarly in the experimental disease, several months after inoculation when clinical signs of infection had disappeared spontaneously without treatment, the red cells and hemoglobin were significantly lower than normal values, although all the other cell elements had returned to normal levels. The only difference observed between the treated and untreated tertiary groups of humans were in the red cells and hemoglobin, both of which were significantly higher in the treated patients. (Red blood cell count—Difference, 480,000 ± 16,100; t, 3.0; P, 0.01. Hemoglobin—Difference, 6.1 ± 2.26 per cent; t, 2.7; P, 0.01.) Thus it would appear that specific

treatment of the tertiary disease in the human causes a rise of the red cell count and hemoglobin value to higher levels, and it is quite probable that similar treatment of the experimental disease would do likewise.

CONCLUSIONS. It has been shown that the treatment of syphilis causes a fall in the total white cell and platelet counts, and the absolute and relative numbers of neutrophils and monocytes, and a rise in the absolute and relative numbers of lymphocytes from values observed in untreated syphilis. That these changes can occur without treatment is evidenced by the fact that changes similar in direction were observed in untreated tertiary syphilis as compared with untreated primary and secondary syphilis. Moreover the same differences were present between the values for treated as compared with untreated early syphilis. One may reasonably conclude that the institution of treatment facilitates the blood changes which occur without treatment if the disease progresses to the tertiary stage. Treatment however accomplishes one additional change in the blood picture, in that it is associated with a rise in the red cell and hemoglobin levels from the low values found in untreated tertiary syphilis.

It has been found that an M/L index higher than 0.55 occurs more frequently in untreated syphilis, and an M/L index lower than 0.55 more frequently in treated cases than would be expected from random association. Moreover, a definite relationship has been found in persistently treated patients between the Wassermann reaction and the values for lymphocytes, monocytes, and the resulting M/L index. The use of the M/L index as a prognostic aid in evaluating the efficacy of treatment is suggested by these findings.

The parallelism in the blood cell changes that exist between the human and the experimental disease in the rabbit, is further evidence of the similarity between the pathologic processes in the two hosts, and lends additional weight to conclusions drawn from experimental syphilis as applied to the human disease.

SUMMARY. From a study of the blood cytology of 126 treated and 87 untreated syphilis patients it was found that:

1. Treatment causes a fall in the total white cell count, the platelet count, and the relative and absolute numbers of neutrophils and monocytes, and a rise in the relative and absolute numbers of lympho-

cytes, and per cent hemoglobin, from values observed in the untreated cases.

2. No significant differences were noted between the mean blood cell values of the primary and secondary untreated patients.

3. A marked anemia was the outstanding feature of the blood cytology in the untreated tertiary disease. This anemia was absent in patients whose treatment was begun in the tertiary stage.

4. No differences in the blood cytology of treated patients were noted when treatment had been begun in the primary, secondary, or tertiary stages of the disease.

5. A monocyte-lymphocyte ratio higher than 0.55 was more frequent among untreated patients, and a monocyte-lymphocyte ratio less than 0.55 was more frequent among treated patients, than would be expected from random association.

6. In a group of persistently treated patients, repeatedly negative serology was more frequently associated with a monocyte-lymphocyte ratio less than 0.45, with lymphocytes higher than 1350 per cc., and with monocytes less than 700 per cc.; and persistently positive serology was more frequently associated with a monocyte-lymphocyte ratio higher than 0.45, with lymphocytes lower than 1350 per cc., and with monocytes higher than 700 per cc. than would be expected from random association.

7. The changes observed in the cytology of the treated patients as compared with the untreated patients were similar in direction and affected the same cells as the changes observed in the spontaneously regressed experimental disease as compared with the period of lesion activity. This similarity lends additional weight to deductions drawn from the experimental disease as applied to human syphilis.

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MOLECULAR ROTATIONS IN MEMBERS OF HOMOLOGOUS SERIES

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Given a substance of type $\mathrm{H{-}\overset{\displaystyle R_1}{\underset{\displaystyle R_2}{\overset{|}{\underset{|}{C}}}}{-}(CH_2)_n}X$, where $n = 0$ or any integer and X any functional group, homologous series may be formed with respect to any of the groups R_1, R_2, or $(CH_2)_nX$. The changes in the rotations of consecutive members will be different in each of the homologous series.

Regarding homologous series with respect to R_2, there are on record a considerable number of observations. Tschugaeff[1] points out that the values of optical rotation for consecutive members increase from member to member in a diminishing ratio until a constant maximum value is reached. Similar observations were made by Rupe.[2] Pickard and Kenyon[3] have recorded in the case of the homologous series of secondary carbinols a certain periodicity in the changes of the numerical values of the rotations of consecutive members. The experience of this laboratory led to the conclusion that in homologous series the shift in the rotation of consecutive members is in the same direction. The values need not uniformly increase or uniformly decrease; on the contrary, in

[1] Tschugaeff, L., *Ber. chem. Ges.*, **31**, 360 (1898); *Chem. Zentr.*, **1**, 93 (1905); *Tr. Faraday Soc.*, **10**, 70 (1914).

[2] Rupe, H., *Ann. Chem.*, **409**, 327 (1915).

[3] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **99**, 45 (1911); **103**, 1957 (1913); **105**, 848, 2233 (1914). See also Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **97**, 380 (1932).

the case where the lower members rotate in the opposite direction from the higher, the values of the consecutive lower members will decrease when those of the higher increase.

The changes in the homologous series with respect to $(CH_2)_nX$ are not quite so simple and from a theoretical view-point are more instructive, particularly with regard to the effect of the distance of a substituent from the asymmetric center on the direction and the value of its contribution to the total rotation of the substance and on its vicinal effect on the other substituents. Tschugaeff was the first to state that the effect of a certain group on the total rotation may disappear when it is removed at a considerable distance from the asymmetric center and Kuhn[4] stressed most emphatically the point that the group CH_2X may become equivalent to CH_3 when removed to a sufficient distance from the asymmetric carbon atom. Thus the substance

$$\begin{array}{c} CH_3 \\ | \\ H\text{—}C\text{—}(CH_2)_nX \\ | \\ (CH_2)_mCH_3 \end{array}$$

should then possess the same optical rotation as the hydrocarbon

$$\begin{array}{c} CH_3 \\ | \\ H\text{—}C\text{—}(CH_2)_{n-1}CH_3 \\ | \\ (CH_2)_mCH_3 \end{array}$$

True, from the equality of the rotations of these 2 molecules, the equality of the contribution of $(CH_2)_nX$ and of $(CH_2)_{n-1}CH_3$ (the number of carbon atoms being the same in both radicles) does not necessarily follow, inasmuch as the rotation of each molecule is the sum of several contributions. An answer to the question of the identity or non-identity of the contributions of these two groups respectively could be obtained from a study of the rotatory dispersion curves of the two substances. However, in the case of certain substances the question may be answered with a great

[4] Kuhn, W., in Freudenberg, K., Stereochemie, Vienna, 404 ff. (1932).

degree of probability, even without a study of the dispersion curves, when they have the structure

$$\begin{array}{c} CH_3 \\ | \\ H—C—(CH_2)_{n-1}CH_2X \\ | \\ (CH_2)_{n-1}CH_3 \end{array}$$

In this case, an equality of the two contributions requires, first, that the value of the rotation of the substance itself should be zero and, second, that the direction of the rotation of the subsequent substance

$$\begin{array}{c} CH_3 \\ | \\ H—C—(CH_2)_nCH_2X \\ | \\ (CH_2)_{n-1}CH_3 \end{array}$$

and the higher members with respect to $(CH_2)_nCH_3$ should be in a direction opposite to that of

$$\begin{array}{c} CH_3 \\ | \\ H—C—(CH_2)_{n-2}CH_2X \\ | \\ (CH_2)_{n-1}CH_3 \end{array}$$

and of the preceding members. From Table I it may be seen that such a condition seems to be attained when X = OH and $n = 3$ or 4 and when R_2 contains as many carbon atoms as the radicle $(CH_2)_{n-1}CH_2OH$. In reality, the zero rotation of the substance is due to the fact that the sum of the contributions of the substituents approaches zero; hence, on bromination they lead to optically active bromides. Undoubtedly, the rotation should become evident in a suitable solvent, or perhaps at a different temperature, and surely at lower wave-length. It is remarkable, however, that the members following that with the zero value rotate in a direction opposite from those that precede them. Thus, it seems to be true that the contribution of the group CH_2OH, after a certain distance from the asymmetric center has been reached, approaches closely the value CH_3 (provided R_2 contains more than 2 carbon atoms), yet never actually becomes equal to it.

For the substances in which X stands for one of the other functional groups given in Table I, a zero value has not yet been obtained. For the case when X represents —COOH, the rotation of the substance becomes small when $n = 3$ and it would be expected to be vanishingly small when $n = 4$. In this connection it is interesting to note that the optical rotations of the acid and carbinol of the structure $H—\overset{CH_3}{\underset{C_2H_5}{C}}—(CH_2)_n\ X$ approached that of the corresponding hydrocarbon after n reached the value 4.

TABLE I—*Maximum Molecular Rotations in Members of Homologous Series of the Type* $H—\overset{CH_3}{\underset{R}{C}}—(CH_2)_nX$, $[M]_D^{25}$ *Homogeneous**

(1)

Structure	X	$[M]_D^{25}$
$H—\overset{CH_3}{\underset{C_2H_5}{C}}—$	$COOC_2H_5$	−22.9
	CH_3	0
	COOH	−18.0
$H—\overset{CH_3}{\underset{(n)C_3H_7}{C}}—$	$COOC_2H_5$	−27.5
	CH_3	0
	COOH	−21.4
$H—\overset{CH_3}{\underset{(n)C_4H_9}{C}}—$	$COOC_2H_5$	−30.7
	CH_3	0
	COOH	−24.3
$H—\overset{CH_3}{\underset{(n)C_5H_{11}}{C}}—$	$COOC_2H_5$	
	CH_3	0
	COOH	
$H—\overset{CH_3}{\underset{(n)C_7H_{15}}{C}}—$	$COOC_2H_5$	−36.5
	CH_3	0
	COOH	−27.3

(2)

Structure	X	$[M]_D^{25}$
$H—\overset{CH_3}{\underset{C_2H_5}{C}}—CH_2$	$COOC_2H_5$	−11.5
	COOH	−10.4
	CH_3	0
	OH	+5.2
	Br	−7.9
$H—\overset{CH_3}{\underset{(n)C_3H_7}{C}}—CH_2$	$COOC_2H_5$	+0.7
	COOH	+3.6
	CH_3	+9.9
	OH	+6.8
	Br	Levo
$H—\overset{CH_3}{\underset{(n)C_4H_9}{C}}—CH_2$	$COOC_2H_5$	+2.9
	COOH	+6.1
	CH_3	+11.4
	OH	+7.9
	Br	Levo
$H—\overset{CH_3}{\underset{(n)C_5H_{11}}{C}}—CH_2$	$COOC_2H_5$	+4.2
	COOH	+8.1
	CH_3	+12.5
	OH	Dextro
	Br	Levo
$H—\overset{CH_3}{\underset{(n)C_7H_{15}}{C}}—CH_2$	$COOC_2H_5$	
	COOH	
	CH_3	
	OH	+11.2
	Br	Levo

(3)

Structure	X	$[M]_D^{25}$
$H—\overset{CH_3}{\underset{C_2H_5}{C}}—CH_2CH_2$	$COOC_2H_5$	−12.7
	COOH	−13.6
	CH_3	−9.9
	OH	−9.1
	Br	−38.8
$H—\overset{CH_3}{\underset{(n)C_3H_7}{C}}—CH_2CH_2$	$COOC_2H_5$	−5.5
	COOH	−6.9
	CH_3	0
	OH	+2.1
	Br	−21.3
$H—\overset{CH_3}{\underset{(n)C_4H_9}{C}}—CH_2CH_2$	$COOC_2H_5$	−2.3
	COOH	−4.1
	CH_3	+1.5
	OH	+4.0
	Br	−16.8
$H—\overset{CH_3}{\underset{(n)C_5H_{11}}{C}}—CH_2CH_2$	$COOC_2H_5$	−0.5
	COOH	−1.9
	CH_3	+2.4
	OH	+6.1
	Br	−14.7

(4)

Structure	X	$[M]_D^{25}$
$H—\overset{CH_3}{\underset{C_2H_5}{C}}—CH_2CH_2CH_2$	$COOC_2H_5$	−13.0
	COOH	−11.1
	CH_3	−11.4
	OH	−11.9
	Br	−21.9
$H—\overset{CH_3}{\underset{(n)C_3H_7}{C}}—CH_2CH_2CH_2$	$COOC_2H_5$	−5.9
	COOH	−3.7
	CH_3	−1.7
	OH	0
	Br	−14.5
$H—\overset{CH_3}{\underset{(n)C_4H_9}{C}}—CH_2CH_2CH_2$	$COOC_2H_5$	−2.8
	COOH	−1.7
	CH_3	0
	OH	+0.7
	Br	−8.3
$H—\overset{CH_3}{\underset{(n)C_5H_{11}}{C}}—CH_2CH_2CH_2$	$COOC_2H_5$	−1.7
	COOH	−0.6
	CH_3	+0.8
	OH	+2.6
	Br	−6.2

(5)

Structure	X	$[M]_D^{25}$
$H—\overset{CH_3}{\underset{C_2H_5}{C}}—CH_2CH_2CH_2CH_2$	$COOC_2H_5$	−13.7
	COOH	−12.2
	CH_3	−12.5
	OH	−12.0
	Br	−14.9
$H—\overset{CH_3}{\underset{(n)C_3H_7}{C}}—CH_2CH_2CH_2CH_2$	$COOC_2H_5$	
	COOH	
	CH_3	−2.4
	OH	−1.7
	Br	−7.8
$H—\overset{CH_3}{\underset{(n)C_4H_9}{C}}—CH_2CH_2CH_2CH_2$	$COOC_2H_5$	
	COOH	
	CH_3	−0.8
	OH	0
	Br	−5.3
$H—\overset{CH_3}{\underset{(n)C_5H_{11}}{C}}—CH_2CH_2CH_2CH_2$	$COOC_2H_5$	
	COOH	
	CH_3	0
	OH	+1.9
	Br	−4.0

(6)

Structure	X	$[M]_D^{25}$
$H—\overset{CH_3}{\underset{C_2H_5}{C}}—CH_2CH_2CH_2CH_2CH_2$	$COOC_2H_5$	
	COOH	
	CH_3	
	OH	−12.5
	Br	−14.0

*The configurations are arbitrary in the sense that they are not correlated to those of the secondary carbinols but they are all correlated among themselves.

In the case of the bromides thus far studied, there is no indication that the value of the contribution of the group CH_2Br is being lowered to that of CH_3, although it is not excluded that, with the increase in the value of n far beyond the values given in Table I, bromides may be obtained with vanishingly small rotations. In the case of chlorides, these conditions should be attained at values of n lower than those required for the bromides.

Further work along the present lines is in progress.

EXPERIMENTAL

4-Methyl Heptanol-1—A Grignard reagent was prepared from 25 gm. of 1-bromo-3-methyl hexane, $[M]_D^{25} = -7.6°$ (homogene-

ous), and 3 gm. of magnesium in ether. To this was added an excess of formaldehyde and the product was allowed to stand overnight. The Grignard reagent was decomposed in the usual way and the carbinol purified through the half phthalic ester. B.p. 81° at 18 mm. Yield 6 gm. No rotation could be detected at 25° in yellow light.

3.246 mg. substance: 8.834 mg. CO_2 and 3.965 mg. H_2O

$C_8H_{18}O$.	Calculated.	C 73.8, H 13.9
130.1	Found.	" 74.2, " 13.7

Levo-4-Methyl 1-Bromoheptane—6 gm. of 4-methyl-1-heptanol, $[M]_D^{25} = 0°$ (from 1-bromo-3-methyl hexane, $[M]_D^{25} = -7.6°$), were cooled in ice and 20 gm. of phosphorus tribromide were added. The product was heated on a steam bath during 30 minutes and then poured onto ice. The halide was purified in the usual way. B.p. 80° at 15 mm. Yield 5 gm. Whereas no rotation could be detected for the carbinol, this bromo compound had considerable optical activity.

$$[\alpha]_D^{24} = \frac{-2.90°}{1 \times 1.075} = -2.7°; \ [M]_D^{24} = -5.2° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{24} = -14.5°$ (homogeneous)

3.230 mg. substance: 5.959 mg. CO_2 and 2.695 mg. H_2O

$C_8H_{17}Br$.	Calculated.	C 49.7, H 8.9
193.1	Found.	" 50 3, " 9.3

Dextro-Ethyl Ester of 3-Methyl Heptanoic Acid-7—20 gm. of 3-methyl heptanoic acid-7, $[M]_D^{26} = +3.56°$ (homogeneous), were dissolved in 50 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid were added. The product was heated on a steam bath during 30 minutes. The excess alcohol was distilled off and the ester extracted with ether. This was washed with sodium carbonate solution, dried with anhydrous sodium sulfate, and then fractionated. B.p. 95° at 25 mm. Yield 20 gm. D 23/4 = 0.865.

$$[\alpha]_D^{23} = \frac{+2.10°}{1 \times 0.865} = +2.43°; \ [M]_D^{23} = +4.18° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{23} = +12.84°$ (homogeneous)

3.090 mg. substance: 7.921 mg. CO_2 and 3.295 mg. H_2O

$C_{10}H_{20}O_2$.	Calculated.	C 69.7, H 11.7
172.2	Found.	" 69.9, " 11.9

Dextro-3-Methyl Heptanol-7—20 gm. of ethyl ester of 3-methyl heptanoic acid-7, $[M]_D^{23} = +4.18°$ (homogeneous), were dissolved in 100 cc. of absolute ethyl alcohol, and this was slowly dropped into a suspension of 40 gm. of sodium in 150 cc. of toluene with vigorous stirring. The carbinol was isolated in the usual way and then purified through its half phthalic ester. B.p. 87° at 20 mm. Yield 12 gm.

$$[\alpha]_D^{24} = \frac{+2.47°}{1 \times 0.825} = +2.99°;\ [M]_D^{24} = +3.90° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{24} = +12.0°$ (homogeneous)
3.150 mg. substance: 8.534 mg. CO_2 and 3.920 mg. H_2O
$C_8H_{18}O$. Calculated. C 73.8, H 13.9
130.1 Found. " 73.9, " 13.9

Dextro-3-Methyl 7-Bromoheptane—10 gm. of 3-methyl heptanol-7, $[M]_D^{24} = +3.84°$ (homogeneous), were cooled in ice and 25 gm. of phosphorus tribromide were added. The product was heated on a steam bath during 30 minutes, cooled, and then poured onto ice. The halide was extracted with ether, purified in the usual way, and then distilled. B.p. 86° at 14 mm. Yield 11 gm.

$$[\alpha]_D^{24} = \frac{+2.76°}{1 \times 1.107} = +2.49°;\ [M]_D^{24} = +4.81° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{24} = +14.92°$ (homogeneous)
4.214 mg. substance: 7.605 mg. CO_2 and 3.280 mg. H_2O
$C_8H_{17}Br$. Calculated. C 49.7, H 8.9
193.1 Found. " 49.2, " 8.7

Dextro-Ethyl Ester of 3-Methyl Octanoic Acid-8—20 gm. of 3-methyl octanoic acid-8, $[M]_D^{27} = 3.93°$ (homogeneous), were dissolved in 50 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid were added. The product was heated on a steam bath during 30 minutes, the excess alcohol distilled off, and water added. The ester was extracted with ether, shaken with sodium carbonate solution, and then distilled. B.p. 110° at 25 mm. Yield 21 gm. $D\ 24/4 = 0.868$.

$$[\alpha]_D^{24} = \frac{+2.05°}{1 \times 0.868} = +2.36°;\ [M]_D^{24} = +4.40° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{24} = +13.66°$ (homogeneous)
5.500 mg. substance: 14.305 mg. CO_2 and 5.970 mg. H_2O

$C_{11}H_{22}O_2$.	Calculated.	C 70.9, H 11.9
186.2	Found.	" 70.9, " 12.1

Dextro-3-Methyl Octanol-8—20 gm. of ethyl ester of 3-methyl octanoic acid-8, $[M]_D^{24} = +4.36°$ (homogeneous), were dissolved in 100 cc. of absolute alcohol and this dropped into a suspension of 40 gm. of sodium in 150 cc. of boiling toluene with rapid stirring. The carbinol was isolated and purified as previously described. B.p. 100° at 20 mm. Yield 12 gm. *D* 24/4 = 0.828.

$$[\alpha]_D^{24} = \frac{+2.30°}{1 \times 0.828} = +2.78°;\ [M]_D^{24} = +4.00° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{24} = +12.5°$ (homogeneous)
3.850 mg. substance: 10.470 mg. CO_2 and 4.730 mg. H_2O

$C_9H_{20}O$.	Calculated.	C 74.9, H 14.0
144.2	Found.	" 74.2, " 13.8

Dextro-3-Methyl 8-Bromooctane—12 gm. of 3-methyl octanol-8, $[M]_D^{24} = +4.0°$ (homogeneous), were cooled in ice and 25 gm. of phosphorus tribromide were added. The product was heated on a steam bath during 30 minutes and then poured onto ice. The halide was extracted and purified as previously described. Yield 14 gm. B.p. 101° at 14 mm. *D* 24/4 = 1.086.

$$[\alpha]_D^{24} = \frac{+2.35°}{1 \times 1.086} = +2.16°;\ [M]_D^{24} = +4.48° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{24} = +13.96°$ (homogeneous)
3.501 mg. substance: 6.704 mg. CO_2 and 3.040 mg. H_2O

$C_9H_{19}Br$.	Calculated.	C 52.2, H 9.3
207.1	Found.	" 52.2, " 9.7

Levo-Ethyl Ester of 2-Propyl Caproic Acid-6—40 gm. of 2-propyl caproic acid-6, $[M]_D^{25} = -0.76°$ (homogeneous), were dissolved in 100 cc. of absolute ethyl alcohol and 5 cc. of concentrated sulfuric acid were added. The product was heated on a steam bath during 30 minutes, the excess alcohol distilled off, and water added. The ester was extracted with ether, shaken with sodium carbonate solution, and then distilled. B.p. 112° at 30 mm. Yield 40 gm. *D* 22/4 = 0.865.

$$[\alpha]_D^{22} = \frac{-0.56°}{1 \times 0.865} = -0.65°;\ [M]_D^{22} = -1.21° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{22} = -5.9°$ (homogeneous)
3.406 mg. substance: 8.769 mg. CO_2 and 3.635 mg. H_2O

$C_{11}H_{22}O_2$.	Calculated.	C 70.9, H 11.9
186.2	Found.	" 70.2, " 11.9

Levo-2-Propyl Hexanol-6—40 gm. of the ethyl ester of 2-propyl caproic acid-6, $[M]_D^{22} = -1.21°$ (homogeneous), were dissolved in 200 cc. of absolute alcohol and this dropped into a suspension of 80 gm. of sodium in 450 cc. of boiling toluene with stirring. The carbinol was isolated and purified as previously described. B.p. 110° at 25 mm. Yield 28 gm. D 24/4 = 0.828.

$$[\alpha]_D^{24} = \frac{-0.42°}{2 \times 0.828} = -0.25°;\ [M]_D^{24} = -0.37° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{24} = -1.72°$ (homogeneous)
3.206 mg. substance: 8.735 mg. CO_2 and 3.910 mg. H_2O

$C_9H_{20}O$.	Calculated.	C 74.9, H 14.0
144.2	Found.	" 74.3, " 13.6

Levo-2-Propyl 6-Bromohexane—20 gm. of 2-propyl hexanol-6, $[M]_D^{24} = -0.36°$ (homogeneous), were cooled in ice and 50 gm. of phosphorus tribromide were added. The mixture was heated on a steam bath during 30 minutes and then poured onto ice. The halide was extracted with ether, the ether distilled off, and the residue purified by treatment with sulfuric acid. B.p. 95° at 12 mm. Yield 22 gm. D 25/4 = 1.082.

$$[\alpha]_D^{25} = \frac{-0.80°}{1 \times 1.082} = -0.74°;\ [M]_D^{25} = -1.53° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{25} = -7.78°$
4.221 mg. substance: 7.994 mg. CO_2 and 3.380 mg. H_2O

$C_9H_{19}Br$.	Calculated.	C 52.2, H 9.3
207.1	Found.	" 51.7, " 9.0

Levo-Ethyl Ester of 2-Butyl Caproic Acid-6—20 gm. of 2-butyl caproic acid-6, $[M]_D^{25} = -0.86°$ (homogeneous), were dissolved in 50 cc. of absolute alcohol and 5 cc. of concentrated sulfuric acid were added. The product was heated on a steam bath during 30 minutes, and then the excess alcohol was distilled off and the ester

isolated in the usual manner. B.p. 125° at 25 mm. Yield 20 gm. D 24/4 = 0.862.

$$[\alpha]_D^{24} = \frac{-1.25°}{2 \times 0.862} = -0.73°;\ [M]_D^{24} = -1.45° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{24} = -2.84°$ (homogeneous)

2.882 mg. substance: 7.579 mg. CO_2 and 3.175 mg. H_2O

$C_{12}H_{24}O_2$.	Calculated.	C 71.9, H 12.1
200.2	Found.	" 71.7, " 12.3

2-Butyl Hexanol-6—40 gm. of ethyl ester of 2-butyl caproic acid-6, $[M]_D^{24} = -1.42°$, were dissolved in 200 cc. of absolute alcohol and this dropped into a suspension of 80 gm. of sodium in 400 cc. of toluene with stirring. The carbinol was isolated and purified as usual. B.p. 125° at 25 mm. Yield 28 gm. D 24/4 = 0.831. No measurable rotation could be detected on the carbinol at 25° in yellow light.

3.506 mg. substance: 9.731 mg. CO_2 and 4.600 mg. H_2O

$C_{10}H_{22}O$.	Calculated.	C 75.9, H 14.0
158.2	Found.	" 75.7, " 14.7

Levo-2-Butyl 6-Bromohexane—20 gm. of 2-butyl hexanol-6 (from ethyl ester of 2-butyl caproic acid-6, $[M]_D^{24} = -1.42°$, homogeneous), were cooled in ice and 50 gm. of phosphorus tribromide were added. The mixture was heated on a steam bath and then poured onto ice. The halide was extracted with ether and purified as previously described. B.p. 112° at 12 mm. Yield 21 gm. D 25/4 = 1.062.

$$[\alpha]_D^{25} = \frac{-1.29°}{1 \times 1.062} = -1.21°;\ [M]_D^{25} = -2.69° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{25} = -5.29°$ (homogeneous)

5.626 mg. substance: 11.225 mg. CO_2 and 4.700 mg. H_2O

$C_{10}H_{21}Br$.	Calculated.	C 54.3, H 9.6
221.1	Found.	" 54.4, " 9.4

Levo-Ethyl Ester of 2-Amyl Caproic Acid-6—20 gm. of 2-amyl caproic acid-6, $[M]_D^{25} = -0.33°$ (homogeneous), were dissolved in 50 cc. of absolute alcohol and 3 cc. of concentrated sulfuric acid were added. The product was heated on a steam bath during 30 minutes and then isolated and purified. B.p. 140° at 25 mm. Yield 21 gm. D 24/4 = 0.864.

$$[\alpha]_D^{24} = \frac{-0.74°}{2 \times 0.864} = -0.43°; [M]_D^{24} = -0.92° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{24} = -1.69°$ (homogeneous)
3.921 mg. substance: 10.475 mg. CO_2 and 4.341 mg. H_2O

$C_{13}H_{26}O_2$.	Calculated.	C 72.8, H 12.2
214.2	Found.	" 72.9, " 12.4

Dextro-2-Amyl Hexanol-6—20 gm. of ethyl ester of 2-amyl caproic acid-6, $[M]_D^{24} = -0.94°$ (homogeneous), were dissolved in 100 cc. of absolute alcohol and this slowly dropped into a suspension of 40 gm. of sodium in 200 cc. of boiling toluene with stirring. The carbinol was isolated and purified as previously described. B.p. 140° at 25 mm. Yield 15 gm. *D* 24/4 = 0.840.

$$[\alpha]_D^{24} = \frac{+1.00°}{2 \times 0.840} = +0.60°; [M]_D^{24} = +1.03° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{24} = +1.85°$ (homogeneous)
3.790 mg. substance: 10.545 mg. CO_2 and 4.640 mg. H_2O

$C_{11}H_{24}O$.	Calculated.	C 76.7, H 14.1
172.2	Found.	" 75.9, " 13.7

Levo-2-Amyl 6-Bromohexane—20 gm. of 2-amyl hexanol-6, $[M]_D^{24} = +1.03°$ (homogeneous), were cooled in ice and 50 gm. of phosphorus tribromide were added. The product was heated on a steam bath during 30 minutes, poured onto ice, and isolated and purified as usual. B.p. 124° at 12 mm. Yield 23 gm. *D* 25/4 = 1.044.

$$[\alpha]_D^{25} = \frac{-0.84°}{1 \times 1.044} = -0.80°; [M]_D^{25} = -1.89° \text{ (homogeneous)}$$

Calculated maximum $[M]_D^{25} = -4.02°$ (homogeneous)
4.350 mg. substance: 9.045 mg. CO_2 and 3.900 mg. H_2O

$C_{11}H_{23}Br$.	Calculated.	C 56.2, H 9.9
235.1	Found.	" 56.7, " 10.0

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ACTION OF NITROUS ACID AND NITROSYL CHLORIDE ON β-PHENYLPROPYLAMINE

A METHOD OF SEPARATING PRIMARY, SECONDARY, AND TERTIARY PHENYL CHLORIDES AND PHENYLCARBINOLS

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The optical rotations of derivatives of the configurationally related methylphenyl- and ethylphenylacetic acids are given in Table I.

On the other hand, Levene, Mikeska, and Passoth,[1] on treatment of the amines prepared from the identical acids with nitrous acid or with nitrosyl chloride obtained carbinols and halides the rotations of which are recorded in Table II.

Thus, the 2-methyl-2-phenylethanol prepared from the amine differed in direction of rotation from that prepared by the reduction of the ester of the identical parent acid. The discrepancy seemed all the more surprising in that the carbinol prepared from the amine was purified through the phthalic acid ester and had the composition required by theory. True, it was not prepared by treatment with nitrous acid but was obtained as a by-product of the treatment with nitrosyl chloride.

In view of this discrepancy the carbinol was now prepared from the amine by treatment with nitrous acid. Contrary to the earlier result, the dextro-methylphenylacetic acid now yielded a levorotatory 2-methyl-2-phenylethanol-1. Thus the acid led to the same carbinol either by the process of reduction of its ester or by deamination of the amine derived from it. For the present we have no definite explanation for the earlier result. *A priori*, two possibilities might have been considered, one being contamination

[1] Levene, P. A., Mikeska, L. A., and Passoth, K., *J. Biol. Chem.*, **88**, 27 (1930).

with a very small quantity of highly active substance, perhaps of its ether, the other that some rearrangement without loss of activity had taken place in the course of the reaction.

TABLE I

Rotation of Phenylated Carboxylic Acids and of Carbinols and Bromides Derived from Them

	$H{-}\overset{CH_3}{\underset{C_6H_5}{C}}{-}COOH$	$H{-}\overset{CH_3}{\underset{C_6H_5}{C}}{-}CH_2OH$	$H{-}\overset{CH_3}{\underset{C_6H_5}{C}}{-}CH_2Br$
$[M]_D^{25}$ (homogeneous)	+111.6°	−20.7°*	−31.0°*
	$H{-}\overset{C_2H_5}{\underset{C_6H_5}{C}}{-}COOH$	$H{-}\overset{C_2H_5}{\underset{C_6H_5}{C}}{-}CH_2OH$	$H{-}\overset{C_2H_5}{\underset{C_6H_5}{C}}{-}CH_2Br$
$[M]_D^{25}$ (homogeneous)	+140°	+2.9°†	−1.8°†

* Maximum rotation (Cohen, J. B., Marshall, J., and Woodman, H. E., *J. Chem. Soc.*, **107**, 900 (1915)).

† Experimental values, not maximum (Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **100**, 688 (1933)).

TABLE II

Rotation of Phenylated Carbinols and Chlorides Derived from Amines of Phenylated Carboxylic Acids (Previously Reported by Levene, Mikeska, and Passoth)*

	$H{-}\overset{CH_3}{\underset{C_6H_5}{C}}{-}COOH$	$H{-}\overset{CH_3}{\underset{C_6H_5}{C}}{-}CH_2OH$	$H{-}\overset{CH_3}{\underset{C_6H_5}{C}}{-}CH_2Cl$
$[M]_D^{25}$ (ether)	+89.5°	+22.1°	−0.5°
$[M]_D^{25}$ (homogeneous)	+96.8°		−12.5°
	$H{-}\overset{C_2H_5}{\underset{C_6H_5}{C}}{-}COOH$	$H{-}\overset{C_2H_5}{\underset{C_6H_5}{C}}{-}CH_2OH$	$H{-}\overset{C_2H_5}{\underset{C_6H_5}{C}}{-}CH_2Cl$
$[M]_D^{25}$ (homogeneous)	+139°	+28°	+17.8°

* Levene, P. A., Mikeska, L. A., and Passoth, K., *J. Biol. Chem.*, **88**, 27 (1930).

The action of nitrosyl chloride on the identical amine resulted, as in the experiment of Levene, Mikeska, and Passoth, in the formation of a levorotatory 2-methyl-2-phenylethyl chloride-1.

Rearrangements on Treatment of 2-Methyl-2-Phenylethylamine with Nitrous Acid and with Nitrosyl Chloride

It was stated above that the formation of a dextrorotatory carbinol under the conditions of the experiment of Levene, Mikeska, and Passoth might have been explained on the basis of a rearrangement to a secondary carbinol, the rearrangement taking place without loss of optical activity. In order to test this possibility, it was necessary to devise a procedure for assaying mixtures composed of the several isomeric carbinols and of a mixture of the isomeric chlorides. The procedures employed were the following.

Carbinols—The primary and secondary phenylated carbinols can be separated from the tertiary by condensation with phthalic anhydride. The tertiary carbinols do not form the ester but are converted into the corresponding unsaturated hydrocarbons. On the other hand, the secondary and tertiary carbinols are differentiated from the primary by their ability to be chlorinated by shaking with hydrochloric acid.

Chlorides—Secondary phenylated chlorides are readily converted into the acetates of the corresponding carbinols by shaking with silver acetate whereas primary chlorides are not, and tertiary are in the main converted into the unsaturated hydrocarbons. On the basis of this property the primary can be freed from the secondary and tertiary chlorides. In order to estimate the proportion of secondary and tertiary chlorides the combined acetates are saponified into the carbinols and from the mixture the secondary carbinol is separated as the acid phthalic ester, which subsequently is saponified yielding the carbinol.

Results

Action of Nitrous Acid—On shaking with hydrochloric acid, a small portion of the carbinols obtained from the amine was chlorinated to about one-third, thus showing that there was present about two-thirds of primary carbinol. The major part was condensed with phthalic anhydride and the carbinol obtained on saponification of the acid phthalic ester was shaken with hydrochloric acid. A chloride did not form by this treatment, thus demonstrating that the carbinol condensed with phthalic anhydride contained no secondary carbinol, and that therefore the product of diazotization consisted of primary and tertiary car-

binols only. The experiment was carried out on the optically active amine and inasmuch as the tertiary carbinol (dimethylphenylmethanol) is optically inactive, the carbinol obtained through the phthalic anhydride was nearly twice as active as the original mixture of carbinols.

Action of Nitrosyl Chloride—On treatment with nitrosyl chloride, a sample of the optically active amine yielded a mixture of chlorides which, on shaking with 0.1 N silver nitrate solution, hydrolyzed to the extent of 10 per cent, thus showing the presence of 90 per cent of the primary halide. The remaining 10 per cent could have consisted either of the tertiary chloride alone, of the secondary alone, or of a mixture of the two. In view of the small proportion of this fraction the experiments dealing with this question were carried out on the inactive base. The secondary and tertiary chlorides were converted into the carbinols by the procedure indicated above and the secondary carbinol was separated from the tertiary by esterification with phthalic anhydride. From the yield of the secondary carbinol the proportion of the secondary chloride was estimated as 3 per cent of the total chlorides thus making the yield of the tertiary to be 7 per cent of the total chlorides.

Nature of the Secondary Chlorides—Two isomeric secondary chlorides may form as a result of the rearrangement in course of the reaction; namely,

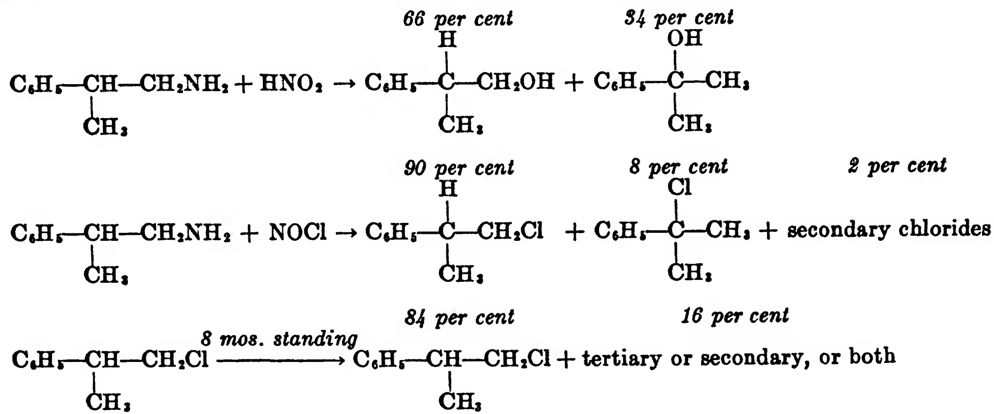
$$\begin{array}{ccc} Cl\text{—}\underset{\substack{|\\ CH_3}}{CH} \cdot CH_2C_6H_5 & \text{and} & Cl\text{—}\underset{\substack{|\\ C_6H_5}}{CH} \cdot CH_2CH_3 \\ \text{I} & & \text{II} \end{array}$$

In a previous article by Levene, Marker, and Rothen,[2] the opinion was expressed that the secondary chloride had the structure (II) on the ground that the substitution of the chlorides by a carboxyl through the Grignard reaction led to a mixture of acids with a dissociation constant corresponding to a mixture of a substituted phenylacetic and a substituted benzylacetic acid. Because of the belief that a tertiary phenylated chloride does not form a Grignard reagent, it was assumed that the substituted phenylacetic acid was derived from substance (II). We now find that the tertiary phenylated chloride can be converted through the

[2] Levene, P. A., Marker, R. E., and Rothen, A., *J. Biol. Chem.*, **100**, 589 (1933).

TABLE III

Proportions of Products Formed in Course of Treatment of Amine with Nitrous Acid and with Nitrosyl Chloride



Grignard reaction into the corresponding disubstituted phenylacetic acid and that therefore our earlier conclusion has to be withdrawn, and hence the nature of the secondary chloride for the present remains uncertain. In view of the small proportion of the secondary chloride formed in the reaction with nitrosyl chloride it would require a considerable supply of the amine in order to solve the problem.

Rearrangement of the 2-Methyl-2-Phenylethyl Chloride-1 on Standing at Room Temperature

Incidentally, it may be mentioned that a sample of 2-methyl-2-phenylethyl chloride which showed no reaction on shaking with 0.1 N aqueous silver nitrate, on standing at room temperature for 8 months, changed in a way to show a loss of 16 per cent of chlorine on shaking at room temperature with this reagent, thus showing rearrangements to the extent of 16 per cent. Whether the product of rearrangement consisted of the tertiary, of the secondary, or of a mixture of the two chlorides remains uncertain for the present.

In Table III are summarized the results of the three reactions described in this paper.

EXPERIMENTAL

Levo-Methylphenylethylamine, $CH_3—\underset{\displaystyle C_6H_5}{\underset{|}{C}H}—CH_2—NH_2$

41 gm. of methylphenylacetonitrile, $[M]_D^{25} = -2.1°$(homogeneous) (from methylphenylacetic acid, $[M]_D^{25} = +18.7°$), were dissolved in 50 cc. of glacial acetic acid and 2 gm. of platinic oxide were added. The mixture was shaken with hydrogen at about 500 mm. pressure until it absorbed no more hydrogen. It was filtered and the excess acetic acid distilled off under reduced pressure. The product was then dissolved in water, extracted with ether, and treated with potassium hydroxide solution. The amine was extracted with ether, the ether evaporated off, and the product distilled. B.p. 85° at 12 mm. $D_4^{25} = 0.949$.

It was shown that at a pressure of 1 atmosphere or below, this amine with glacial acetic acid and platinic oxide is not reduced to the cyclohexyl derivative with hydrogen.

$$[\alpha]_D^{25} = \frac{-3.31°}{1 \times 0.949} = -3.49°; \quad [M]_D^{25} = -4.71° \text{ (homogeneous)}$$

4.125 mg. substance:	12.080 mg. CO_2 and 3.535 mg. H_2O		
86.2 " "	required 6.19 cc. 0.1 N HCl		
$C_9H_{13}N$.	Calculated.	C 79.9, H 9.7, N 10.4	
	Found.	" 79.9, " 9.6, " 10.1	

Action of Nitrous Acid on Levo-Methylphenylethylamine—20 gm. of methylphenylethylamine, $[M]_D^{25} = -4.6°$ (homogeneous), were dissolved in twice the theoretical amount of 10 per cent sulfuric acid cooled in ice. 20 gm. of sodium nitrite in a small amount of water were slowly added with stirring and the solution allowed to stand 3 hours. The product was heated during 1 hour at 60°, cooled, and extracted with ether. The ether extract was dried and the product distilled. B.p. 75–95° at 4 mm., showing a mixture. $\alpha = -0.91°$ (homogeneous).

3.325 mg. substance:	9.740 mg. CO_2 and 2.690 mg. H_2O	
$C_9H_{12}O$.	Calculated.	C 79.4, H 8.9
	Found.	" 79.9, " 9.1

5 gm. of the above product were shaken with 35 per cent aqueous hydrochloric acid. The product was extracted with ether, washed with water, and then distilled. The distillate was analyzed for chlorine. As only secondary phenylcarbinols and tertiary phenylcarbinols react with cold aqueous hydrochloric acid, this reaction permits the estimation of the amount of the combined tertiary and secondary carbinols present. Analysis showed the product to contain 8.85 per cent of chlorine, or about one-third tertiary and secondary carbinols.

The remainder of the carbinol was treated with phthalic anhydride by the usual procedure. The phthalate was purified, then hydrolyzed to the carbinol by means of potassium hydroxide solution. This carbinol boils sharply at 92° at 4 mm. Yield 10 gm.

$$[\alpha]_D^{25} = \frac{-1.75°}{1 \times 0.998} = -1.75°; \quad [M]_D^{25} = -2.39° \text{ (homogeneous)}$$

The above procedure separates primary and secondary from tertiary carbinols as the latter do not form phthalates.

5.760 mg. substance: 16.815 mg. CO_2 and 4.570 mg. H_2O

$C_9H_{12}O$.	Calculated.	C 79.4, H 8.9
	Found.	" 79.6, " 8.9

A 5 gm. lot of the above carbinol purified through its phthalic ester was shaken with aqueous hydrochloric acid for 30 minutes. It was extracted with ether, washed with water, and then distilled. B.p. 92° at 4 mm. The specific rotation was unchanged. $[\alpha]_D^{25} = -1.75°$.

The product was analyzed for chlorine. There was no chlorine present, showing that the original product of the treatment of the methylphenylethylamine with nitrous acid gives about one-third tertiary and two-thirds primary carbinols and no secondary carbinol.

Action of Nitrosyl Chloride on Levo-Methylphenylethylamine—20 gm. of methylphenylethylamine, $[M]_D^{25} = -4.6°$ (homogeneous), were dissolved in 100 cc. of dry ether. The solution was cooled in carbon dioxide and alcohol, and a slight excess of nitrosyl chloride in dry ether was slowly added. The ether was evaporated off on a steam bath and the crude product distilled under reduced pressure. The distillate was shaken with a small amount of phosphorus pentoxide and then redistilled. B.p. 71° at 2 mm. $[M]_D^{25} = -1.2°$ (homogeneous).

5.895 mg. substance: 15.166 mg. CO_2 and 3.672 mg. H_2O
6.145 " " : 5.650 " AgCl

$C_9H_{11}Cl$.	Calculated.	C 69.9, H 7.2, Cl 22.9
	Found.	" 70.2, " 7.1, " 22.7

12 gm. of the above chloride were converted into the next higher acid by means of the Grignard reagent and carbon dioxide. This gave a levo acid as previously reported.[3]

4.435 mg. substance: 11.895 mg. CO_2 and 2.960 mg. H_2O

$C_{10}H_{12}O_2$.	Calculated.	C 73.1, H 7.4
	Found.	" 73.2, " 7.5

Separation of Primary, Secondary, and Tertiary Phenyl Chlorides—100 gm. of methylphenylethylamine were treated with nitrosyl chloride in ether at −50°. The chlorides were isolated as described before. Yield 50 gm.

8.410 mg. substance: 7.500 mg. AgCl
$C_9H_{11}Cl$. Calculated, Cl 22.9; found, Cl 22.1

A test sample was shaken with silver nitrate solution and the quantities of secondary plus tertiary chlorides estimated in this manner. This showed 10 per cent. The total solution was shaken during 3 hours with 2 liters of silver acetate solution. The product was extracted with ether and the ether evaporated. The residue was then shaken with cold aqueous potassium hydroxide solution to hydrolyze the acetates formed. During this process some of the primary chloride likewise was hydrolyzed to the carbinol. The product was extracted with ether. This solution contained the unchanged primary chloride, some primary carbinol, the secondary carbinol, and possibly traces of tertiary, and the unsaturated hydrocarbon derived from the tertiary chloride. The entire solution was treated with phthalic anhydride. The phthalates were extracted with sodium carbonate. The unesterified fraction then contained the primary chloride and the unsaturated hydrocarbon. These two were extracted with ether and the ethereal solution fractionated. This gave 37 gm. of unchanged chloride and 3 gm. of unsaturated hydrocarbon.

3.924 mg. substance: 13.164 mg. CO_2 and 3.020 mg. H_2O

C_9H_{10}.	Calculated.	C 91.5, H 8.5
	Found.	" 91.5, " 8.6

The phthalates were hydrolyzed with potassium hydroxide solution giving 6 gm. of carbinol which were shaken with aqueous hydrochloric acid and analyzed for chlorine. As the secondary phenylcarbinols react with cold aqueous hydrochloric acid and the primary carbinols do not, the percentage of secondary carbinol present can be determined.

6.909 mg. substance: 0.880 mg. AgCl

$C_9H_{11}Cl$.	Calculated.	Cl 22.9 (for complete chlorination)
	Found.	" 3.2

This showed about 14 per cent of secondary chloride in the hydrolyzed products, or 0.9 gm. of recovered secondary chloride from 50 gm. of starting material. As the original material contained 10 per cent secondary plus tertiary halides, the approximate proportions of chlorides formed on treatment of the amine with nitrous acid were

Primary	chlorides................................	90 per cent
Secondary	"	2 " "
Tertiary	"	8 " "

Rearrangement in 2-Methyl-2-Phenylethyl Chloride-1 on Standing—A sample of the pure primary chloride which gave no test for secondary or tertiary chloride (prepared by halogenation of the carbinol) was allowed to stand at room temperature during 8 months. At the end of that time it contained 16 per cent of tertiary or secondary chloride, or both.

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SERINEPHOSPHORIC ACID OBTAINED ON HYDROLYSIS OF VITELLINIC ACID. II

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The study undertaken in this laboratory of the so called phosphopeptones of vitellin and of casein had for its object a more comprehensive aim than the mere isolation of the amino acid combined with the phosphoric acid residue. Our aim was to study the amino acids which grouped around the phosphoric acid ester, since in different phosphoproteins these groups might have a different composition. There was reason to believe that such was the case inasmuch as vitellinic acid, which may be regarded as the prosthetic group of vitellin, contains a very large proportion of basic components and apparently none at all or a very small proportion of dicarboxylic acids (Posternak and Posternak,[1] Lipmann and Levene[2]); whereas casein peptone, having a ratio of N:P identical with that of vitellinic acid, contains a large proportion of dicarboxylic acids (Rimington[3]). It seemed possible that the stability of the phosphopolypeptides in each case was due to the presence of the phosphoric acid residue in one or more of the amino acids composing the polypeptide. On the basis of this consideration it seemed reasonable to expect that on phosphorylation all proteins would behave similarly to the natural phosphoproteins and hence by the aid of phosphorylation it was expected to obtain information as to the amino acids which, in different proteins, group around the hydroxy acids.

In the case of casein, Schmidt,[4] and Levene and Hill[5] have

[1] Posternak, S., and Posternak, T., *Compt. rend. Acad.*, **184**, 909 (1927); **187**, 313 (1928).

[2] Lipmann, F. A., and Levene, P. A., *J. Biol. Chem.*, **98**, 109 (1932).

[3] Rimington, C., *Biochem. J.*, **21**, 272, 1179 (1927).

[4] Schmidt, G., *Sunti comunicaz. scient., XIV cong. internaz. fisiol.*, Rome, 228 (1932).

[5] Levene, P. A., and Hill, D. W., *J. Biol. Chem.*, **101**, 711 (1933).

demonstrated that the amino acid immediately connected with serinephosphoric acid is glutamic acid. The product of hydrolysis of vitellinic acid by means of 2 N hydrochloric acid had the same composition as that of casein peptone as far as the ratio of N:P was concerned, this being 1.3 to 1.5 in each case. The substance with the ratio of 1.0 described by Lipmann and Levene has now been obtained on further hydrolysis of the product with the higher N:P ratio. It seemed, therefore, that the product of the first hydrolysis would yield a phosphodipeptide in the case of vitellinic acid by the method of fractionation of the brucine salts, as in the case of casein peptone. This expectation, however, was not realized inasmuch as, in the case of vitellinic acid, serinephosphoric acid was the only product which could be isolated in pure state from the brucine salts of the product of the first hydrolysis. Two crystallizations of the crude brucine salts from methanol led to the pure product.

It was subsequently found that a practically pure barium salt of serinephosphoric acid could be obtained directly from the product of the first hydrolysis of vitellinic acid by causing the salt to settle out from its aqueous solution by bringing the solution to a boil and filtering the precipitate while hot. This salt could be purified by redissolving in cold water and repeating the above procedure. From the viewpoint of yield, however, it was found more advantageous to use a combined method; namely, separating the barium salt of the phosphoserine from the crude products by boiling of the aqueous solution, converting the salt obtained in this manner into the brucine salt, and reconverting this into the barium salt.

By this method a sufficient quantity of material became available to permit the isolation of serine from the product of hydrolysis of the barium salts. This amino acid was obtained in analytically pure state on first crystallization. Bearing in mind that serine is one of the most soluble amino acids, it is justified to conclude that no other amino acid was present among the products of hydrolysis of the barium salt.

CONCLUSIONS

1. Definite proof is furnished for the conclusion that the substance described by Lipmann and Levene is the barium salt of phosphoserine.

2. It was shown that from vitellinic acid under milder conditions of hydrolysis a phosphoric ester of serine is obtained, whereas from casein under more rigorous treatment a phosphoric ester of a dipeptide composed of serine and glutamic acid is obtained.

3. The latter circumstance leads to the further conclusion that in vitellin the serinephosphoric acid is not linked to one of the dicarboxylic amino acids.

EXPERIMENTAL

Isolation of d-Serinephosphoric Acid

First Method—The hydrolysis of vitellinic acid was carried out under the conditions described by Lipmann and Levene;[2] namely, by heating a suspension of the substance in 10 volumes of 2 N hydrochloric acid for 10 hours on the boiling water bath under reflux. The solution was cooled, made faintly alkaline to phenolphthalein, and filtered.

The filtrate was rendered exactly neutral by the addition of sulfuric acid and again filtered. This filtrate was then concentrated under reduced pressure to a small volume (30.0 gm. of vitellinic acid to a volume of 200 cc.) and an equal volume of 98 per cent alcohol added. The precipitate thus formed was centrifuged and washed repeatedly with 50 per cent alcohol until the wash solution contained only a trace of barium chloride. The precipitate was dissolved in cold water (hot water should not be used) and reprecipitated with an equal volume of 98 per cent alcohol. After a few washings the precipitate was generally free of barium chloride and contained no inorganic phosphates. This precipitate was again dissolved in cold water, the barium ions were removed quantitatively by means of sulfuric acid, and the filtrate was rendered faintly alkaline by means of a methyl alcoholic solution of brucine. The excess of brucine was removed by shaking the solution with chloroform and the aqueous solution was concentrated to dryness under reduced pressure. The residue was dissolved in the minimum quantity of boiling methanol. On cooling, a crystalline deposit was formed. The mother liquor concentrated to dryness formed a small second deposit (Fraction II) of crystals heavier than those of the first deposit (Fraction I). The two substances differed in their melting points and in the respective ratios of N:P.

The yield from 60 gm. of vitellinic acid was 60 gm. of brucine salt of Fraction I and 9.0 gm. of Fraction II.

Each fraction was twice recrystallized from methanol. There was no significant change in the melting points, the substance of Fraction I having a melting point of 98° (decomposing at 105°) and the substance of Fraction II having a melting point of 120° (decomposing at 130°).

The composition of the substance of Fraction I was approximately that of the dibrucine salt of serinephosphoric acid.

4.915 mg. substance: 0.305 cc. N_2 at 24.5° and 755 mm.
3.445 " " : 7.515 mg. $(NH_4)_3PO_4$, 14 MoO_3

$C_{49}H_{60}N_5O_{14}P$.	Calculated.	N 6.27, P 2.77
	Found.	" 7.07, " 3.22

The composition of the substance of Fraction II was not much different from that of Fraction I. On conversion into the barium salt, however, it was found to consist of a mixture of substances with the average content of N, 6.63 per cent and of P, 3.27 per cent.

For conversion into the barium salts the brucine salts were dissolved in cold water and a cold aqueous solution of barium hydroxide was added so long as a precipitate of brucine continued to form. The precipitated brucine was removed by filtration and the traces remaining in solution were removed by extraction with chloroform. The remaining aqueous solution was rendered exactly neutral by addition of sulfuric acid, and the barium sulfate was removed by filtration. The filtrate was concentrated to a very small volume and the barium salt precipitated by means of an equal volume of 98 per cent alcohol.

The composition of the barium salt of Fraction I was as follows:

6.710 mg. substance: 2.583 mg. CO_2 and 1.365 mg. H_2O
9.400 " " : 0.308 cc. N_2 at 27° and 758.5 mm.
4.549 " " : 27.800 mg. $(NH_4)_3PO_4$, 14 MoO_3
28.905 " " : 19.950 " $BaSO_4$

$C_3H_6O_6NPBa$.	Calculated.	C 11.2, H 1.82, N 4.36, P 9.65, Ba 42.7
	Found.	" 10.5, " 2.27, " 4.12, " 8.93, " 40.60

Second Method—The crude barium salts were dissolved in a small volume of cold water with the aid of a very little acetic acid. The solution was then rendered neutral with aqueous ammonia,

filtered, and the filtrate brought to a boil. At this point an amorphous white precipitate formed which was filtered off while hot. The precipitate was washed with small portions of boiling water and then with alcohol of increasing concentration, beginning with 50 per cent alcohol.

Dried to constant weight, the substance had the following composition.

9.293 mg. substance: 0.335 cc. N_2 at 35° and 754 mm.
4.595 " " : 25.185 mg. $(NH_4)_3PO_4$, 14 MoO_3

$C_3H_6O_6NPBa$.	Calculated.	N 4.36,	P 9.65,	N:P 1.0
	Found.	" 3.92,	" 8.03,	" 1.0

Following this experience, the method adopted for the preparation of *d*-serinephosphoric acid was modified in the following way. The crude phosphates still containing traces of barium chloride were taken up in a small volume of boiling water and the mixture kept boiling with stirring for 10 minutes. The insoluble fraction was removed by filtration and, on addition of an equal volume of 98 per cent alcohol to the filtrate, a second precipitate formed. This precipitate, which still contained a small proportion of barium chloride, was freed from the latter by the procedure given above. From 60 gm. of vitellinic acid there were obtained 11.0 gm. of the heat-insoluble fraction and 3.5 gm. of the second fraction. The heat-insoluble fraction was then dissolved in cold water and the brucine salts prepared as given above.

Hydrolysis of the Barium Salt of d-Serinephosphoric Acid

7.0 gm. of the barium salt were dissolved in cold water and the barium ions removed quantitatively by means of sulfuric acid. Sufficient hydrochloric acid was added to make the total concentration of this acid 20 per cent and the total volume 200 cc. The solution was boiled under reflux for 24 hours, after which the chloride ions were removed by means of silver sulfate. The filtrate from silver chloride was treated with hydrogen sulfide. The sulfuric and phosphoric acids were then removed by an excess of barium hydroxide, and the excess of barium hydroxide by means of sulfuric acid. The solution was then concentrated to dryness. The residue was taken up in a little hot water. On cooling there was formed a crystalline deposit consisting of heavy prisms. The

yield was 0.350 gm. On addition of an equal volume of 98 per cent alcohol a second crop of 0.350 gm. was obtained from the mother liquor.

The composition of the substance was as follows:

4.798 mg. substance:	6.060 mg. CO_2 and 2.870 mg. H_2O	
5.676 " "	: 0.664 cc. N_2 at 32° and 759 mm.	
$C_3H_7O_3N$.	Calculated.	C 34.28, H 6.73, N 13.33
	Found.	" 34.54, " 6.70, " 13.08

The rotation of the substance was

$$[\alpha]_D^{23} = \frac{-0.58° \times 100}{4 \times 1} = -14.5°$$

The filtrate from the second precipitate gave a very small third fraction which was not further analyzed. Thus, the total yield was about 0.800 gm. or about 50 per cent of the theoretical. Decomposition of the serine to pyruvic acid is undoubtedly responsible for a considerable part of the loss.

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THE CULTIVATION OF MONOCYTES IN FLUID MEDIUM

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Plate 29

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The purpose of the experiments described in this article was to ascertain the conditions under which monocytes could be cultivated in a fluid medium. The plasma coagulum that is customarily used in the Carrel technique[1] for the cultivation of these cells seriously interferes with quantitative measurements of their metabolism. For such studies, it would be of great advantage to replace it with a liquid. That monocytes can live for a time in fluid has been shown by the work of Lewis and Lewis,[2] and also that of de Haan.[3] In the experiments of Lewis and Lewis,[2] the monocytes lived in hanging drops of liquefied plasma for 2 to 4 weeks, feeding partly, at least, on the other cells of the blood. In de Haan's experiments,[3] they were cultivated for 2 to 3 weeks in a slowly flowing stream of peritoneal exudate. De Haan[3] has already made some measurements of their metabolism under these conditions. The fact that his technique requires very large volumes of fluid limits its usefulness to a certain extent. Moreover, peritoneal exudate does not contain all the ingredients necessary for the prolonged cultivation of monocytes. De Haan[3] supplemented its nutritive properties by the addition of polymorphonuclear leucocytes from time to time. In the experiments described in this article, an effort was made to find a means whereby monocytes could be cultivated

[1] Carrel, A., *J. Exp Med.*, 1923, **38,** 407. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, **36,** 365; 1926, **44,** 285.

[2] Lewis, M. R., *Am. J. Path.*, 1925, **1,** 91. Lewis, M. R., and Lewis, W. H., *J. Am. Med. Assn.*, 1922, **84,** 798. Lewis, W. H., *Harvey Lectures*, 1925–26, **21,** 77. Lewis, W. H., *Arch. exp. Zellforsch.*, 1928, **6,** 253.

[3] de Haan, J., *Bull. histol.*, 1927, **4,** 293; *Arch. exp. Zellforsch*, 1928, **6,** 276; 1930–31, **10,** 82. de Haan, J., Kolk, K. H., and Gerritsma, H., *Arch. exp. Zellforsch.*, 1928, **7,** 283; 1929, **8,** 452.

for a longer time in a small volume of fluid without using other cells as food.

Plan of the Experiments

The experiments of Carrel and Ebeling[1] have indicated that monocytes cultivated in coagulated plasma feed upon the serum rather than the fibrin. Yet, up to the present, all attempts to grow them in serum without a fibrin substratum have failed. It seemed probable that this was due to the fact that in a coagulum acid, and perhaps other metabolites, accumulate around the cell colony, thus creating the conditions necessary for its survival, while in the absence of a coagulum, the products of metabolism are quickly removed from the vicinity of the cells.

As early as 1913, Rous[4] showed by adding litmus to the medium that sarcoma and embryonic tissue embedded in coagulated plasma gave rise to enough acid within a few hours to turn blue litmus red in the immediate vicinity of the tissue. This has since been found to be true of monocytes. When measured with the glass electrode of MacInnes and Dole,[5] it was found that the pH of the medium immediately surrounding a piece of chick embryo spleen dropped in half an hour from 8.0 to 7.2. In 24 hours, it fell to 6.8, and in several days to 6.2.[6] Aside from this acid production, very little is definitely known about the changes in the medium surrounding a colony of monocytes.

The problem was approached, therefore, by ascertaining first the pH at which chicken monocytes would survive longest in serum. For this, homologous serum at 25 per cent concentration in Tyrode solution was used and adjusted by means of lactic acid to pH values ranging from 6.0 to 7.8. Then, a comparison was made of the relative value of using lactic acid, hydrochloric acid, and carbon dioxide for regulating the acidity. Finally, serum at the optimum pH value was modified by the addition of a variety of substances in an attempt to find a medium in which the monocytes would live, and to reproduce, if possible, the conditions that exist around a colony of

[4] Rous, P., *J. Exp. Med.*, 1913, **18**, 183.

[5] MacInnes, D., and Dole, M., *J. Gen. Physiol.*, 1929, **12**, 805.

[6] Baker, L. E., unpublished experiments.

monocytes embedded in plasma. Glutathione was used because of its reducing action, and its rôle in cell respiration.[7] Proteins hydrolyzed to varying degrees by enzymes were tried, because it is known that monocytes will digest particles of protein which they have ingested,[8] and also because hydrolyzed proteins have been found to increase enormously the proliferation rate of monocytes[9] when incorporated in their medium. Particles of denatured protein, coagulated egg white, and dead muscle, which monocytes feed upon,[8] were also used. Various combinations of nucleic acid, hemoglobin, glutathione, and amino acids or digested proteins were tried, since they have been found beneficial in the building up of a synthetic medium for fibroblasts.[10]

Technique

For these experiments, the following technique was used. A tiny fragment of chick embryo spleen or of blood leucocytic film was fastened to the bottom of a Carrel D-3 flask by means of a drop of 25 per cent plasma. The plasma was spread over about 2 sq. cm. of the surface of the flask, and coagulated with a trace of tissue juice. 1.5 cc. of experimental fluid was then added. This procedure allowed the cells to form a colony instead of scattering throughout the whole medium, and made it possible to measure their rate of migration for several days. At the same time, this small coagulum was so very thin that the composition of the medium within it could not differ appreciably from that of the fluid as a whole. After a few days, the original fragment was extirpated from the clot. Later, when quite a number of cells had migrated onto the glass, or had been deposited there from the fluid, the tiny clot and the cells it contained were also removed. In this way, any possible effects of the original tissue and of the coagulum were eliminated. From this time on, the entire medium was liquid.

The changes in hydrogen ion concentration of the medium were followed in each culture day by day. In some experiments, this was done by removing a drop of the medium and testing it on a drop plate with indicators. In other experiments, phenol red at a concentration of 0.005 per cent was incorporated in the medium, and comparison made with standard flasks containing the same amount of phenol red in buffers at known pH values.

Adjustment of the pH of the medium with lactic or hydrochloric acids was made just before adding the fluid to the culture. With CO_2, it was accomplished by

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[7] Hopkins, F. G., *Biochem. J.*, London, 1925, **19**, 787.

[8] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, **44**, 285.

[9] Baker, L. E., *J. Exp. Med.*, 1933, **57**, 689.

[10] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, **47**, 353. Baker, L. E., *J. Exp. Med.*, 1929, **49**, 163.

replacing the air of the culture flask with a gas mixture containing 21 per cent oxygen, sufficient carbon dioxide to give the desired pH value, and nitrogen. This method has been used now for some time to adjust the pH of cultures made with coagulated plasma. With the fluid cultures, the gas was introduced immediately, and contained a higher percentage of carbon dioxide in order to lower the pH value to a greater degree. The concentration of carbon dioxide necessary was determined, of course, by the concentration of serum and other buffering substances in the medium, as well as by the pH desired.

EXPERIMENTAL RESULTS

Effect of the Hydrogen Ion Concentration.—It was found that the monocytes proliferated most rapidly when the initial pH of the medium was 7.2 to 7.4. As long as the pH was not allowed to fall below 7.0 or 6.8, the cells remained clear, active, free from dark granulations, and showed no tendency to degenerate (Fig. 1). They remained in excellent condition, even after the original tissue and the small coagulum had been removed from the flask.

Effect of Different Acids.—Equally good results were obtained with all three acids (lactic, hydrochloric, and carbon dioxide), both in respect to the rate of proliferation and the condition of the cells. Adjustment with carbon dioxide was adopted as the routine procedure, however, since it does not destroy the buffer action of the medium, as the other acids do, and is also more convenient to use.

Effect of the Concentration of Serum.—Increasing the concentration of serum in the medium caused an increase in the rate of proliferation of the monocytes. In some sera, this continued up to 100 per cent concentration. Other sera, however, appeared to be toxic at concentrations above 50 per cent. In 25 per cent serum, multiplication was slow, but the cells remained active and relatively clear, with small cytoplasmic structures. In 50 per cent serum, the monocytes proliferated more rapidly. They also became larger and contained more and larger granules and fat globules. In very high concentrations of serum, they ultimately deteriorated.

Effect of Added Metabolites or Nutrients.—None of the metabolites or nutritive materials added to the serum improved its value as a culture medium for the monocytes. Glutathione, nucleic acid, hemoglobin, and sodium lactate had no beneficial action at the concentrations tried in these experiments. Particles of egg yolk, denatured

albumin, and heated muscle were ingested by the cells, as is usual in a coagulum,[6] but the cells became large, round, dark, and granular, and appeared inert. The products of tryptic digestion of protein increased the rate of proliferation of the monocytes, as they do in plasma,[9] but were not necessary to their life. The cells became more fatty, and larger numbers of them died when the digestion products were added to the medium. On the other hand, serum alone appeared to be entirely adequate for the nutrition of the monocytes.

Description of Colonies Cultivated for 2 Months in Serum

Several colonies of monocytes, both from blood and from spleen, have been cultivated now for over 2 months in 25 per cent and also in 50 per cent serum (Fig. 2). They are still as active as at the beginning of the experiment and give every evidence from their appearance that life and proliferation will continue indefinitely, if the same conditions of cultivation are maintained. Throughout this time, the medium was adjusted to an initial pH of 7.4. A gas mixture containing 3 per cent CO_2 was used with 25 per cent serum, and one containing 5 per cent CO_2 with 50 per cent serum. New medium was given when the pH had fallen to 7.0 or 6.8. In those colonies in which the pH was allowed to fall below this value without renewing the medium, the cells became larger and more granular (Fig. 3). They also accumulated large refractile globules and showed a tendency to adhere to each other. They did not die even when the pH fell to 6.4 or somewhat below. At first, when only a small number of cells was present, renewal of the medium once in 4 days was sufficient. Later, new serum was supplied every 24 hours. After a time, so many cells accumulated in the flask that a portion of them had to be removed to prevent the pH from falling below the desired value.

Most of the cells adhered firmly to the glass surface of the flask. Those that did come loose were deposited on the glass at a distance from the colony and started a new colony. The lymphocytes and polymorphonuclear leucocytes that were present at the beginning soon disappeared, as they do in coagulated plasma. The fibroblasts from the spleen grew out less rapidly than the wandering cells and were

removed when the original fragment and tiny coagulum were extirpated. Therefore, a pure strain of monocytes was obtained. Instead of scattering and migrating away from each other, as they do in coagulated plasma, the monocytes, both in the tiny clot and on the glass, remained in close proximity. As multiplication proceeded, they spread out in a monocellular layer, covering the entire surface of the flask and almost touching each other. The appearance (Figs. 1 and 2) of the individual cells was entirely like that already described by Lewis and Lewis[2] in their studies of monocytes adhering to the cover-slip in hanging drop cultures. They flattened out on the glass in a more or less circular form (Figs. 1 and 2), presenting a very different appearance than they do as they migrate through a fibrin coagulum (Fig. 4).

The small monocytes were gradually transformed into macrophages, as always occurs in cultures of these cells when they are well fed. Some giant cells were formed, as has also been reported by Lewis and Lewis,[2] and de Haan.[3] Smaller monocytes adhered to these giant cells and appeared to be sucked into them, or to unite with them, just as Lewis[2] has described. In these experiments, however, the number of giant cells was very small in comparison with the number of normal macrophages.

The cells continued to proliferate throughout the entire period of cultivation. In less than a month, the whole surface of the flask was covered with them. To test their continued ability to proliferate, the cells on one-half of the surface of the flask were scraped off with a platinum spatula. These, together with the medium, were transferred to another flask. New medium was then added to both flasks, and cultivation continued. The cells that had been suspended in the fluid soon adhered to the surface of the new flask and continued to proliferate. In 2 to 3 weeks, the entire surface of this flask was covered with cells. The monocytes in the original flask also increased in number so that the half of the flask that had been freed from cells was again covered with them in a short time. In order to continue their cultivation, large areas of cells were removed from the flask every few days. Otherwise, too great a decrease in the pH value of the medium or exhaustion of its food constituents would have occurred.

Subcultivation of Cells Suspended in the Fluid

Though the great majority of monocytes adhered firmly to the glass, a few of them were usually to be found in the fluid when it was removed. That these cells were alive and in good condition was shown by cultivating them again in a coagulum. The fluid was centrifuged and the few cells it contained were taken up in a drop of plasma containing some embryonic juice. This was transferred to a new flask and allowed to coagulate. Enough dilute plasma was then added to cover the rest of the surface of the flask, and the pH was brought down immediately to 7.2 with carbon dioxide. After a few days, the cells migrated into the outer coagulum. Fig. 4 shows cells cultivated in this way after 12 days' sojourn in fluid medium.

DISCUSSION

In the experiments described in this paper, it has been shown that monocytes can be cultivated in pure strain in a fluid medium. Serum diluted with Tyrode solution supplied all the necessary food material. At the end of 2 months' cultivation, the cells were still in excellent condition, multiplying rapidly, and giving every indication that they would continue to proliferate indefinitely under the same conditions. Cultivation in dilute serum without a coagulum was made possible by immediately reducing the pH of the medium to 7.4 or 7.2, and not allowing it to fall below 7.0 or 6.8 during incubation. Monocytes embedded in a coagulum at a higher pH value survive only because their combined metabolism lowers the pH around them to this value or a lower one. The cells die in a fluid in which it is not possible for them to lower the pH to a sufficient extent. It seems probable that the monocytes cultivated in the hanging drop cultures of Lewis and Lewis[2] lived because the conditions of those experiments either did not permit so much loss of CO_2 from the medium as takes place in the Carrel flasks, or because the number of cells present was great enough to reduce the pH of the single drop of medium to a value that permitted their survival. De Haan[3] does not lay very great stress on the hydrogen ion concentration required for cultivation of monocytes in his flowing medium. In some experiments, he saturated the fluid with 7 per cent CO_2. In others, the original pH value of the medium was 8.0.

He states, however, that the pH in the chambers containing the cells varied from 7.2 to 6.8. This agrees well with the present experiments. Even if the original pH was 8.0, a large number of cells in a small chamber could reduce it to 7.2, especially if it were not well buffered. Cultivation in Carrel flasks at higher pH values than those used in these experiments might be successful, if enough cells were present to lower the pH quickly, or if the medium had a smaller buffer value. In this case, new serum would have to be supplied more often.

In the first days of cultivation, the monocytes undoubtedly fed to some extent on the other cells present. However, cultivation was continued so long after the lymphocytes and polymorphonuclear leucocytes disappeared that it was evident that these other cells are not needed for food if fresh serum is supplied fairly often. Moreover, monocytes transferred to a new flask, after a pure strain had been obtained, continued to proliferate in serum without any other food material.

No attempt has been made as yet to cultivate isolated or even a very small number of cells under these conditions. It is quite possible that single cells would require still different conditions from those described here.

SUMMARY

Monocytes from blood and from spleen have been cultivated in fluid medium in Carrel flasks for over 2 months. Diluted serum supplied all the essential nutritive substances. Cultivation in fluid was made possible by adjusting the initial pH of the fluid to 7.4, and not allowing it to fall below 7.0 or 6.8. The cells remained in good condition when the pH was adjusted with either lactic acid, hydrochloric acid, or carbon dioxide. Adjustment with carbon dioxide was found to be more convenient and also more practical, since it does not destroy the buffer action of the medium. After 2 months of cultivation, the monocytes were in excellent condition and still proliferated actively. They gave every indication that indefinite multiplication could be maintained under the conditions of these experiments. It is hoped that this method of cultivation, with some modifications, will prove useful in studying the metabolism of these cells.

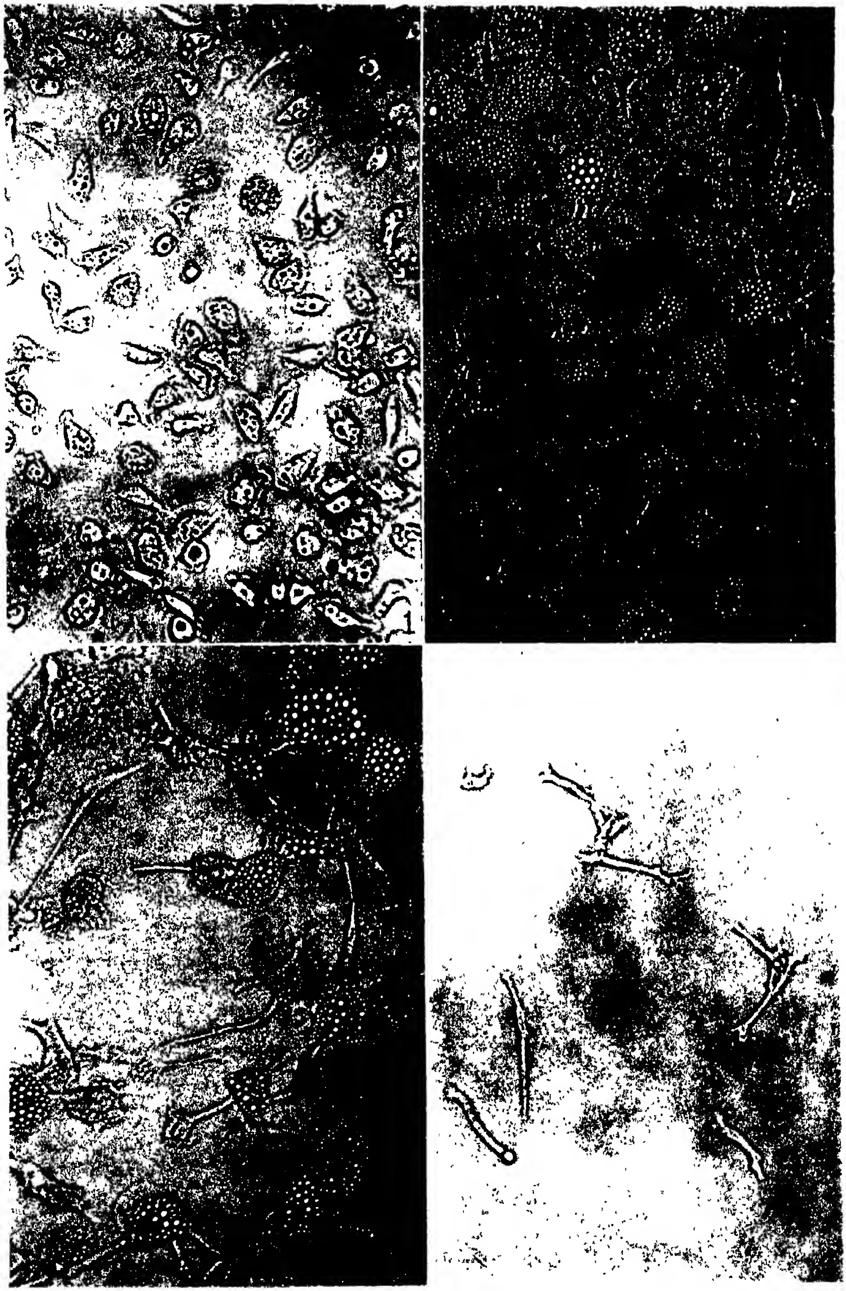
EXPLANATION OF PLATE 29

FIG. 1. Monocytes from chick embryo spleen cultivated in 25 per cent serum for 30 days. The medium was adjusted initially to pH 7.4 with carbon dioxide, and not allowed to fall below 7.0 during cultivation. ×230.

FIG. 2. Monocytes from chick embryo spleen cultivated in 50 per cent serum for 64 days. The pH fell below 6.8 several times during cultivation. ×230.

FIG. 3. Monocytes from chick embryo spleen cultivated in 50 per cent serum for 2 months. So many cells were present that the pH fell repeatedly below 6.8 during 24 hours' incubation. Consequently, the cells are loaded with granulations and show a tendency to agglutinate. × 230.

FIG. 4. Monocytes obtained from the fluid of a culture, which had been 12 days in liquid medium, now being cultivated in a coagulum. ×230.



(Baker: Monocytes in fluid medium)

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THE ESTIMATION OF TRYPSIN WITH HEMOGLOBIN

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The method for the estimation of trypsin described in this paper is essentially the same as our method for the estimation of pepsin (Anson and Mirsky, 1932). Trypsin is allowed to digest denatured hemoglobin in a slightly alkaline phosphate solution. Precipitation of the denatured hemoglobin by the phosphate or by salt added with the enzyme is prevented by urea. The undigested hemoglobin is precipitated with trichloracetic acid. The amount of digested hemoglobin not precipitated, which is a measure of the amount of trypsin used, is estimated by the blue color which the tyrosine and the tryptophane in the digested hemoglobin give with the phenol reagent. Cysteine and heme (even heme whose iron is in the ferric state) also can reduce the phenol reagent. But there is very little cysteine in hemoglobin and all the heme is precipitated with trichloracetic acid.

The procedure has several advantages. Many estimations can be made in a short time; the results are entirely reproducible; the hemoglobin solution keeps for at least a month without change; and the rate of digestion is not sensitive to considerable amounts of acid, alkali, urea or glycerol added with the enzyme.

The procedure has two disadvantages. In the first place, the hemoglobin solution cannot be used for the estimation of the active native trypsin in a mixture of active native and inactive denatured trypsins because inactive denatured trypsin changes into active native trypsin in the hemoglobin solution just as it does in all the protein solutions which have hitherto been used for the estimation of trypsin. This change can be prevented by making the hemoglobin solution more alkaline as is described in a following paper. In the second place,

the activity of a crude pancreatic extract is higher when measured by the digestion of hemoglobin than when measured by the change in the viscosity of gelatin. The reasons for this are being studied. In the experiments with purified trypsin so far carried out the two methods yield the same results.

Commercial dried proteins can be used instead of hemoglobin prepared in the laboratory. They are of dubious reproducibility and they contain considerable and variable amounts of color-producing substances not precipitated by trichloracetic acid.

When gelatin, casein or any other non-reproducible protein substrate is used for the estimation of trypsin by any method, the procedure can be calibrated by means of a solution of purified trypsin whose activity has been measured by the hemoglobin method which yields reproducible results. The calibration curve states the extents to which a particular sample of protein is digested under given conditions by different known amounts of trypsin. A sufficiently purified trypsin can be prepared from commercial trypsin in a few minutes by a modification of the Northrop-Kunitz procedure (1932) which avoids several filtrations. A solution of this partially purified trypsin which digests hemoglobin at the same rate as a solution of crystalline trypsin also has the same effect on the viscosity of gelatin as does the crystalline trypsin.

The Procedure.—1 ml. of enzyme solution is added to 5 ml. of the hemoglobin solution to be described later. The 175 × 20 mm. test-tube containing the 6 ml. of digestion mixture is whirled and placed in a water bath at 25°C. After 5 minutes 10 ml. of 5 per cent trichloracetic acid are poured in from another test-tube, the suspension is poured back and forth, allowed to stand 5 minutes and filtered. To 5 ml. of filtrate are added 10 ml. of 0.50 N sodium hydroxide and 3 ml. of the phenol reagent of Folin and Ciocalteau (1927) diluted three times (*cf.* Wu, 1922, and Greenberg, 1929). The reagent is added drop by drop with stirring and is always added in the same way. After 1 to 10 minutes the blue color is read against the color developed from 0.00083 milliequivalents (0.15 mg.) of tyrosine dissolved in 5 ml. of 0.2 N hydrochloric acid.

If the trichloracetic acid suspension is filtered immediately instead of after 5 minutes the first half of the filtrate contains some undigested hemoglobin in fine suspension and this first portion must accordingly be rejected or refiltered. Centrifugation can be used instead of filtration without any difference in results.

Preparation of Tyrosine Standard.—The tyrosine is thrice crystallized and its concentration is estimated by Kjeldahl (100 mg. tyrosine = 7.74 mg. nitrogen).

It is stored at room temperature in 0.2 N hydrochloric acid containing 0.5 per cent formaldehyde. Some preservative is needed to prevent the destruction of tyrosine by mould even in the cold. Formaldehyde does not affect the color value of tyrosine.

Copper sulfate solution or a blue glass inserted in the plunger of the colorimeter can be used as a standard instead of the blue solution obtained from tyrosine. Although these standards do not match the tyrosine blue in white light they do match it in the fairly monochromatic red light transmitted by the Corning Glass Filter No. 241.

Rubber, even after being boiled with alkali, contains reducing substances which can be extracted by the reagents so all contacts with rubber should be avoided.

Preparation of the Hemoglobin Solution.—Defibrinated bovine blood is centrifuged, the serum and white corpuscles are siphoned off and the red corpuscles are washed once with an equal volume of 0.9 per cent sodium chloride solution.[1] Water is added to give a solution containing in 100 ml. 10.5 gm. hemoglobin or 1.86 gm. nitrogen. This solution is stored frozen in paraffined paper ice-cream containers.

To denature the hemoglobin and to remove substances not precipitated with trichloracetic acid which give a color with the phenol reagent, one proceeds as follows. A mixture of 220 ml. 10.5 per cent hemoglobin and 11 ml. 1 N sodium hydroxide is brought to 50–60°C. and is added to 1300 ml. of water previously brought to 100°C. There is then added with mechanical stirring 26 ml. of a solution 5 M in respect to sodium chloride and 0.5 M in respect to KH_2PO_4. The resulting suspension is filtered on a folded paper, the precipitate is washed with water, transferred to a beaker, weighed and enough water added to make the weight 400 gm. 400 gm. of urea are then stirred up with the precipitate and 160 ml. of 1 N sodium hydroxide are added. After solution of the protein and the urea 200 ml. of 1 M KH_2PO_4 plus 240 ml. water are added. The solution is stored in the cold with toluol as a preservative. The solution is the same as would be obtained by adding 40 gm. of urea to 100 gm. of a solution which contains 2.2 gm. denatured hemoglobin (about 5 per cent of the protein is lost) and the equivalents of 100 ml. of 0.2 M KH_2PO_4 and 80 ml. of 0.2 M sodium hydroxide.

Preparation of Solutions of Commercial Dried Proteins.—25 gm. of edestin (La Roche) hemoglobin (Eimer and Amend) or casein (after Hammarsten) are mixed with 400 gm. urea. This mixing facilitates the solution of the protein. In the case of casein and edestin, the protein and urea are simply put together in a

[1] These washed corpuscles can be stored frozen and after being dialyzed and acidified can be used for the estimation of pepsin instead of the purified hemoglobin solution already described (Anson and Mirsky, 1932) which is more difficult to prepare. The one acid hemoglobin solution which we have prepared from frozen corpuscles was digested at the same rate as the purified hemoglobin.

flask which is whirled. In the case of hemoglobin the protein and urea are ground together in a mortar. 240 ml. water and 160 ml. 1 N sodium hydroxide are added to the urea-protein mixture and the solution is brought to room temperature. After the protein is dissolved (and denatured) 200 ml. 1 M KH_2PO_4 and 375 ml. water are added. Eimer and Amend's hemoglobin dissolves more readily than other commercial hemoglobins we have tried. Although it is labelled pure it is contaminated with other proteins. Of the three proteins, edestin is the most rapidly digested. A sample of the Hoffman-La Roche edestin, however, was not digested at the same rate as crystalline edestin prepared in the laboratory.

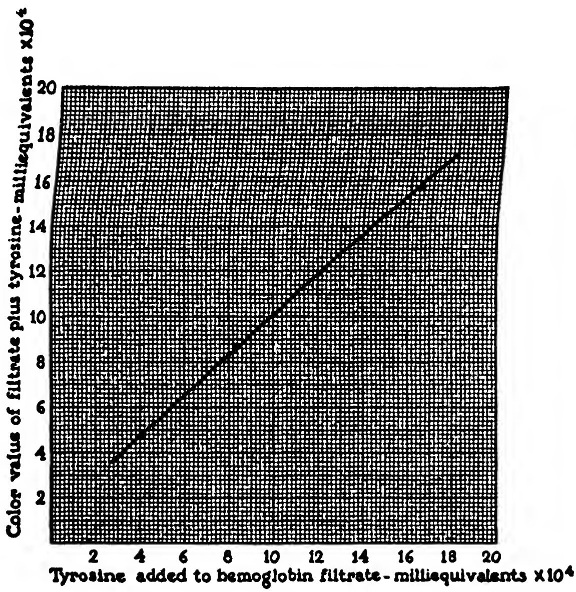

FIG. 1. Color values of various amounts of tyrosine dissolved in hemoglobin filtrates.

Calculations

What is measured is the color value of 5 ml. of the trichloracetic acid filtrate from digested hemoglobin in terms of the amount of tyrosine which would give the same color under the same conditions. For the purposes of calibration it must, therefore, first be determined how much color would be given by various known amounts of tyrosine in the trichloracetic acid filtrate from undigested hemoglobin which contains in addition to trichloracetic acid, phosphate and urea a small amount of color-producing substance not precipitated by trichloracetic acid.

10 parts 5 per cent trichloracetic acid are added to a mixture of 5 parts hemoglobin solution and 1 part water. To 5 ml. portions of the filtrate are added 1 ml. portions of 0.1 N hydrochloric acid containing various amounts of tyrosine. The colors developed with sodium hydroxide and the phenol reagent are read against the color developed from 0.15 mg. or 0.00083 milliequivalents, tyrosine dissolved in 5 ml. 0.2 N hydrochloric acid plus 1 ml. water. Fig. 1 shows how many

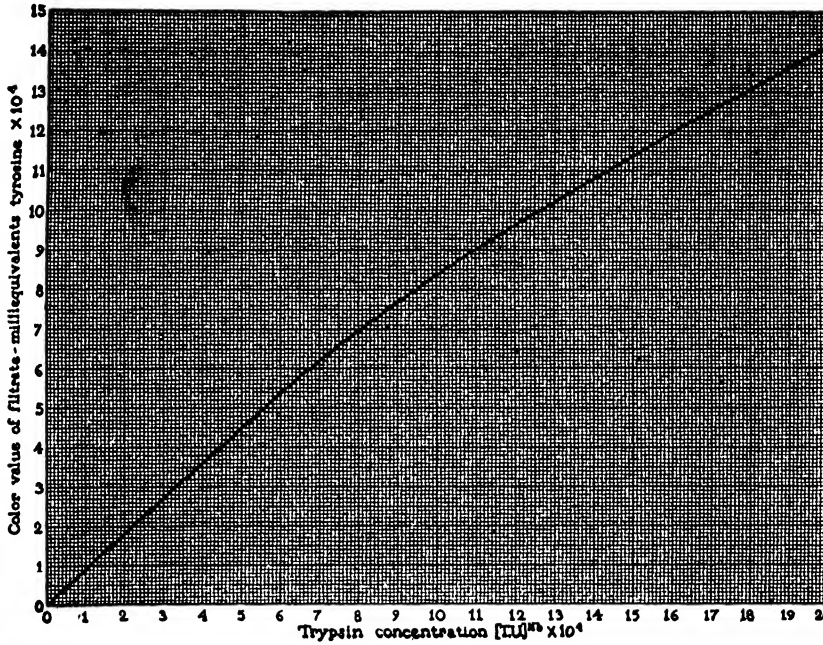

FIG. 2. Relation of trypsin concentration to color value of digestion products, 5 ml. filtrate, 5 minutes digestion at 25°C.

milliequivalents of tyrosine in hydrochloric acid are needed to give the same color as any given amount of tyrosine dissolved in the trichloracetic acid filtrate.

Digestion is now carried out with various amounts of enzyme. Fig. 2 gives the color values of the filtrates in terms of the amounts of tyrosine in the filtrate which would give the same colors. In practice since the properties of the hemoglobin solution are constant one avoids calculations by using a curve in which the amounts of trypsin

are plotted directly against the colorimetric readings when the standard is set at 20. One does not have to use different calibration curves if different periods of digestion are used because increasing the diges- tion time n times is always equivalent to increasing the enzyme concentration n times.

For the purpose of using the hemoglobin-urea procedure the trypsin units may be considered as arbitrary numbers which are proportional to the amounts of trypsin which give the amounts of color-producing substances expressed by the curve. In order, however, to make the hemoglobin trypsin unit comparable with other units of proteolytic activity (Northrop, 1932; Anson and Mirsky, 1932) the following definition has been adopted. One unit of trypsin produces in 1 minute at 35.5°C. in 6 ml. of the digestion mixture an amount of color-producing substance not precipitable with trichloracetic acid which gives the same color as 1 milliequivalent of tyrosine. This definition assumes that the extent of digestion is proportional to the concentration of enzyme and to the time of digestion. These assumptions are correct only when the amount of digestion is small since as digestion proceeds the trypsin is inhibited by the products of digestion. The slope of the curve of Fig. 2 for small amounts of digestion is $1/5 \times 16/5 \times 1.75$ or 1.12 times less steep than it would be if the determination were carried out as described in the definition because the digestion is carried out for 5 minutes instead of 1, only 5 ml. of filtrate are used in the colorimetric estimation instead of the total 16 and the digestion is carried out at 25°C. instead of at 35.5°C. at which it is 1.75 times faster.

Effect of Variations in the Composition of the Digestion Mixture on the Extent of Digestion

Hemoglobins from the bloods of different individual animals are digested at the same rate. Doubling the hemoglobin concentration or reducing it 10 per cent has no detectable effect. The amount of urea can be increased or decreased 5 per cent, or the equivalent of 1 ml. of 0.1 N hydrochloric acid, 0.1 N sodium hydroxide or 10 per cent glycerol can be added to the digestion mixture without changing the extent of digestion 3 per cent.

Preparation of Purified Trypsin for the Standardization of Non-

Reproducible Proteins.—1 gm. of Fairchild's trypsin is suspended in 25 ml. 0.1 N hydrochloric acid, heated for 1 minute at 80°C. and cooled rapidly to room temperature with ice water. After 10 minutes, 6 gm. of ammonium sulfate are added and the suspension filtered. To each 10 ml. of the filtrate are added 2 gm. ammonium sulfate. The resulting precipitate is centrifuged and dissolved in enough 0.005 N hydrochloric acid to make the final volume 25 ml. This final solution has about 0.01 activity unit per ml.; *i.e.*, it has to be diluted about 10 times for estimation.

SUMMARY

The formation from hemoglobin of split products not precipitable by trichloracetic acid is taken as a measure of tryptic activity. The split products are estimated colorimetrically.

Many measurements of tryptic activity can be made in a short time and different samples of hemoglobin yield the same results.

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THE ESTIMATION OF ACTIVE NATIVE TRYPSIN IN THE PRESENCE OF INACTIVE DENATURED TRYPSIN

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Trypsin, which can catalyze the hydrolysis of proteins, is itself a protein (Northrop and Kunitz, 1932). The denaturation of proteins is reversible (Anson and Mirsky, 1931). When trypsin is denatured its proteolytic activity is completely lost. When the denaturation of trypsin is reversed the original proteolytic activity is completely restored (Northrop, 1932). The denaturation of trypsin and its reversal can therefore be followed by measurements of proteolytic activity. The present paper describes a technique for the measurement of the activity of native trypsin in the presence of inactive, denatured trypsin. Later papers will describe the application of this technique to the study of the effect of various denaturing agents such as heat, acid, alcohol and urea on the equilibria between native and denatured trypsin and on the rates of the denaturation of trypsin and its reversal.

To estimate trypsin the enzyme is added to a solution of protein and the rate of digestion is measured. In all the protein solutions which have hitherto been used reversal of the inactivation and denaturation of trypsin take place. If active, native trypsin is to be estimated in the presence of inactive, denatured trypsin, such change of inactive into active trypsin during the very estimation must obviously be avoided. This we have succeeded in doing by adding a suitable amount of the denaturing agent, urea, to the digestion mixture.

We have already described a method for the estimation of trypsin with hemoglobin (Anson and Mirsky, 1933) which can be used when the reversal of inactivation need not be considered. Digestion of

denatured hemoglobin by trypsin is allowed to take place in a slightly alkaline solution buffered with phosphate. Precipitation of the denatured hemoglobin is prevented by urea. The undigested hemoglobin is precipitated with trichloracetic acid. The digested hemoglobin not precipitated by trichloracetic acid, which is a measure of the amount of trypsin used, is estimated by the blue color it gives with the phenol reagent. Considerable amounts of acid, alkali or glycerol can be added with the enzyme without any effect on the rate of digestion.

The change of inactive into active trypsin which takes place in the standard hemoglobin solution used for the ordinary estimation of trypsin can be prevented by adding more urea or (as is done in the procedure to be described) more alkali. If too much urea or too much alkali is added not only is the change from inactive to active trypsin prevented but the active trypsin is inactivated so fast that no measurement of activity is possible. In a digestion mixture in which there is neither reactivation nor a too rapid inactivation the rate of digestion is slower than it is in the standard hemoglobin solution used for the ordinary estimation of trypsin and the rate of digestion is much more sensitive to small changes in pH and urea concentration.

The general method of preventing reactivation during an analytical procedure by having present an inactivating agent such as urea may prove to be of use not only in the study of the denaturation of pure proteins but in testing for the protein nature of biologically active substances which have not been isolated but which are present in extremely dilute solutions together with many impurities. One would expect a protein substance regardless of its concentration or of the presence of impurities to lose its activity if exposed to denaturation procedures or to the proteolytic activity of trypsin. Trypsin, itself, however, which is a protein, can be heated in acid to 100°C. without any permanent loss of activity, for the denaturation and inactivation which take place on heating are reversed on cooling. There are proteins like hemoglobin which in their native form are not attacked by trypsin although in their denatured form they are rapidly digested. If a substance does not lose its activity when exposed to a denaturation procedure or to trypsin it may mean, there-

fore, not that the substance is not a protein but that there has been, as in the case of trypsin, reversal of denaturation or that, as in the case of hemoglobin, the protein must be denatured to be digested by trypsin. To make sure that there has been no inactivation by denaturation the activity should be measured under conditions which prevent the reversal of denaturation. To make sure that the substance is not digestible by trypsin the substance should first be exposed to conditions known to cause denaturation and then brought to the slightly alkaline solution suitable for tryptic digestion in the presence of some substance such as urea which will keep the substance denatured.

The Procedure.—5 ml. of the hemoglobin solution to be described are poured from a 175 × 20 mm. test-tube into 1 ml. of the enzyme solution in a second tube, the solution is poured back and forth, and the two test-tubes are placed in a water bath at 25°C. Sometime during the digestion period the small amount of solution in the test-tube from which the digestion mixture was last poured is drained into the other test-tube. After 5 minutes 10 ml. 5 per cent trichloracetic acid are added, the suspension is mixed with the few drops of digestion mixture still left in the third tube, allowed to stand 5 minutes and filtered. To 5 ml. of filtrate are added 10 ml. of 0.50 N sodium hydroxide and 3 ml. of the phenol reagent (Folin and Ciocalteau, 1927) diluted three times are added dropwise with stirring and always in the same way. After 1 to 10 minutes the blue color is read against the color developed from 0.00083 milliequivalents (0.15 mg.) of tyrosine dissolved in 5 ml. of 0.2 N hydrochloric acid containing 0.5 per cent formaldehyde. If the colorimetric comparison is made in the fairly monochromatic red light transmitted by the Corning glass filter No. 241 almost any blue glass or a blue solution can be used as a standard.

Preparation of the Hemoglobin Solution.—Defibrinated bovine blood is centrifuged, the serum and white corpuscles are siphoned off and the red corpuscles are washed once with an equal volume of 0.9 per cent sodium chloride solution. Water is added to give a solution containing in 100 ml. 10.5 gm. hemoglobin or 1.86 gm. nitrogen. This solution is stored frozen in paraffined paper ice-cream containers.

150 ml. of 10.5 per cent hemoglobin plus 7.5 ml. 1 N sodium hydroxide at 50–60°C. are added to 900 ml. of water at 100°C. There is then added with mechanical stirring 18 ml. of a solution 5 M in respect to sodium chloride and 0.5 M in respect to KH_2PO_4. The precipitate is filtered, washed and made up to 390 gm. with water. 390 gm. of urea and then 20 cc. 1 N sodium hydroxide are added and the solution is brought to room temperature to insure the complete solution of the hemoglobin. Finally, 160 ml. of 0.5 M boric acid and 430 ml. water are added and the solution is stored at 5°C. with toluol as a preservative. The

solution is the same as would be obtained by adding 39 gm. of urea to 100 gm. of a solution containing 1.5 gm. denatured hemoglobin and the equivalents of 80 ml. 0.1 M boric acid and 20 ml. 0.1 M sodium hydroxide.

Calibration.—Digestion is carried out with various dilutions of a trypsin solution whose activity in terms of trypsin units has been measured by the standard

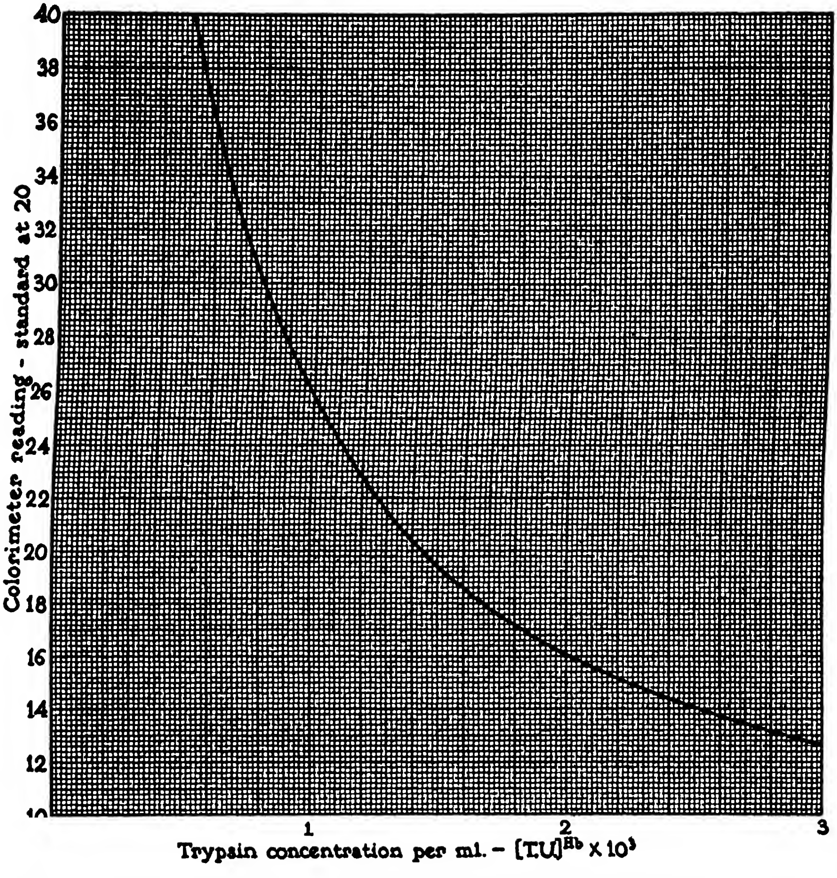

FIG. 1. Relation of trypsin concentration to color value of digestion products.

hemoglobin method. The colors developed from 5 ml. of trichloracetic acid filtrate are read against the color developed from 5 ml. of the standard tyrosine solution. Fig. 1 gives the relation between the trypsin units and the colorimetric readings when the standard is set at 20. The hemoglobin solution may be kept at least 2 months without any change in the calibration curve and different preparations of the hemoglobin solution yield the same calibration curve.

The Effect of Variations in the Composition of the Digestion Mixture on the Rate of Digestion.—Increasing the urea concentration 5 per cent reduces the extent of digestion 2 per cent. Decreasing the urea concentration 5 per cent increases the extent of digestion 2 per cent. The addition to the digestion mixture of the equivalent of 1 ml. of 5 per cent glycerol causes an increase of 6 per cent in the digestion; 1 ml. of 0.06 N hydrochloric acid causes an increase of 4 per cent; and 1 ml. of 0.06 N sodium hydroxide causes a decrease of 6 per cent. If more than 0.01 N acid or alkali is added with the enzyme then 0.5 ml. instead of 1 ml. of enzyme solution is used and there is added to the 5 ml. of hemoglobin solution 0.5 ml. of a solution which exactly neutralizes the acid or alkali added with the enzyme.

Evidence of the Prevention of the Reversal of Denaturation.—When trypsin is heated to 60°C. in 0.01 hydrochloric acid for 2 minutes it is completely inactivated and denatured. On cooling the solution the original activity is restored. If 5 ml. of the hemoglobin solution plus 0.5 ml. of water are poured into 0.5 ml. of a hot 0.01 N hydrochloric acid solution of trypsin which before heating contained 24×10^{-3} units of active enzyme and digestion is carried out for 10 minutes only 0.65×10^{-3} units of activity are found.

When trypsin is heated or cooled to 45°C. in 0.01 N hydrochloric acid it is about half inactivated and denatured. The activity of such a mixture is the same whether or not the denatured half of the trypsin is first precipitated with salt and removed. The experiment is carried out as follows: Into 0.5 ml. of trypsin (1.72×10^{-2} $[\text{T.U.}]^{\text{Hb}}$) in 0.01 hydrochloric acid heated to 45°C. for 5 minutes are poured 5 ml. hemoglobin solution plus 0.5 ml. water. 1 ml. of the mixture is immediately added to 5 ml. of a mixture of 5 parts hemoglobin solution and 1 part water. The digestion is carried out for 10 minutes after the hemoglobin solution was first poured into the enzyme solution. For the estimation with salt, 2 ml. of 5 M sodium chloride in 0.01 N hydrochloric acid are poured into 2 ml. of the heated trypsin solution. The resulting suspension is centrifuged. 1 ml. of the supernatant solution is added to 5 ml. hemoglobin solution; 1 ml. of this mixture is then immediately added to 5 ml. of a mixture of 5 parts hemoglobin solution to 1 part water and the digestion is carried

out as before. By both methods the heated solution is found to have 0.86×10^{-2} units of activity.

Effect of Variations in the Composition of the Digestion Mixture on the Extent of the Reversal of Inactivation.—The less urea in the digestion mixture the more reversal of inactivation. If the amount of urea is decreased 5 per cent the amount of reversal is still less than 5 per cent. If the amount of urea is decreased a third about 20 per cent reversal takes place. The results under these conditions are not very reproducible. Acid favors reversal, alkali the reverse, but the addition of 1 ml. of 0.04 N hydrochloric acid or sodium hydroxide (which is more than permissible if the rate of digestion by active trypsin is to be kept constant) has no significant effect on the extent of reversal. Glycerol favors reversal. The addition of the equivalent of 1 ml. of 5 per cent glycerol to the digestion mixture increases the amount of reversal 60 per cent.

SUMMARY

Inactive denatured trypsin changes into active native trypsin in the protein solutions which have been used to estimate tryptic activity. If the digestion mixture, however, is alkaline enough and contains enough urea this change does not take place. Such a digestion mixture can be used to estimate active native trypsin in the presence of inactive denatured trypsin.

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THE EQUILIBRIUM BETWEEN ACTIVE NATIVE TRYPSIN AND INACTIVE DENATURED TRYPSIN

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Hemoglobin denatured by acid or coagulated by heat and dissolved in acid becomes native again when brought to nearly neutral solution. From this fact the conclusion was drawn that there is an equilibrium between native and denatured protein which depends on the temperature and the pH (Anson and Mirsky, 1925). If there is such an equilibrium then at a suitable pH denaturation brought about by heating should be reversed by cooling. Reversal of heat denaturation by mere cooling was actually found to take place with serum albumin, globin, and pepsin (unpublished experiments). In none of these three cases was reversal complete, so none of these three proteins was suitable for the exact study of definite equilibrium states. A suitable protein has now been found. Trypsin, which catalyzes the hydrolysis of proteins, is itself a protein (Northrop and Kunitz, 1932). And the denaturation of trypsin is readily and completely reversible. If trypsin is heated to 60°C. in 0.05 N acid it is converted into a protein which is completely precipitable by quarter saturated ammonium sulfate. When the heated trypsin is cooled it changes back into the original form which is not precipitated by quarter saturated ammonium sulfate. If trypsin is heated or cooled to a temperature around 40°C. a definite fraction is precipitable by salt (Northrop, 1932).

Since native trypsin digests other proteins and denatured trypsin does not, the denaturation of trypsin can be followed by activity measurements. If the digestion mixture is alkaline enough and contains enough urea then there is no change of inactive denatured trypsin into active native trypsin during the measurement of tryptic

activity. The same results are obtained whether denaturation is followed by measurements of activity with the urea technique or by measurements of the formation of salt-precipitable protein. In the present investigation the urea technique alone has been used both because of its simplicity and because it can be applied even to dilute solutions of trypsin. For the definition of trypsin activity units ($[T. U.]^{Hb}$) and the details of the procedure a previous paper (Anson and Mirsky, 1933) should be consulted. Hemoglobin is used as the protein substrate which is digested.

TABLE I

Effect of Acid, Alkali, and Alcohol on the Equilibrium between Native and Denatured Trypsin

Composition of solvent	Temperature of inactivation	$[T. U.]^{Hb}$ per ml. before heating $\times 10^4$	$[T. U.]^{Hb}$ after heating and cooling $\times 10^4$	$[T. U.]^{Hb}$ when heated to temperature of inactivation $\times 10^4$	$[T. U.]^{Hb}$ when cooled to temperature of inactivation $\times 10^4$	Percentage inactivation
	°C.					
0.01 N HCl	44.1	21.6	21.4	11.4	11.0	48
0.01 N HCl	44.0	199.2		99.6		50
0.003 N HCl	54.5	28.8	27.6	14.7	13.2	51.5
0.001 N HCl	61.3	28.8	28.4	14.4	15.2	48.4
0.01 N HCl in 10 per cent alcohol	38.5	28.8	28.4	14.4	14.7	49.4
0.05 N NaOH	0	345.0	260.0	17.4		95.0

Existence of Equilibria.—Under any definite conditions under which there is no irreversible inactivation, a definite fraction of the trypsin is in the active, native form and a definite fraction in the inactive, denatured form. The percentage inactivation at a given temperature is the same whether the trypsin solution is heated or cooled to that temperature (*cf.* Table I). In general the percentage denaturation at equilibrium depends only on the conditions at equilibrium and is the same whether one starts with native or denatured trypsin.

Effect of Concentration.—The concentration of a trypsin solution can be varied ten times without any effect on the percentage denaturation under given conditions (*cf.* Table I). The kinetics of denaturation are therefore the same as the kinetics of the reversal of denaturation.

Effect of pH.—In a rough way the more trypsin is ionized by either acid or alkali the more the equilibrium between native and denatured trypsin is shifted toward the denatured form. A theory of the mechanism of denaturation by acid and alkali will be presented in a paper on the equilibrium between native and denatured hemoglobin. According to this theory the effect of pH on the equilibrium between the native and denatured forms of a protein must be correlated with differences in the titration curves of the native and denatured forms. The data at present available do not permit any detailed comparison of the effects of pH on the ionization and denaturation of trypsin.

The marked effect of pH on the equilibrium between native and denatured trypsin is not apparent at room temperature. At 25°C. native trypsin is the equilibrium form even in 0.01 N hydrochloric acid. That is, denatured trypsin, if brought to 25°C. in 0.01 N hydrochloric acid, changes completely into native trypsin. The importance of pH is clear at higher temperatures. If trypsin is heated at any pH a temperature is reached at which the enzyme is half denatured. This temperature of half denaturation is very sensitive to the pH and is lowered by either acid or alkali (*cf.* Table I). Only in acid solution can the experiments be carried out without irreversible inactivation. In acid solution, as is shown in Table I, the activity after heating and cooling is the same as before heating. In alkaline solutions in which the temperature of half denaturation is low there is irreversible inactivation. In neutral solutions in which the temperature of half denaturation is high, the denatured trypsin is digested so rapidly by the native trypsin that measurements of equilibria are impossible. The irreversible inactivation by alkali and the digestion in neutral solution have been studied in detail by Northrop and Kunitz (1934).

The Effect of Temperature.—In 0.01 N hydrochloric acid trypsin is almost completely native at 40°C. and almost completely denatured at a temperature 10° higher. The logarithm of the equilibrium constant (the ratio of native trypsin to denatured trypsin) is proportional to the reciprocal of the absolute temperature as is stated by van't Hoff's relation between the heat of reaction and the effect of temperature on the equilibrium

$$\ln K = -\frac{\Delta H}{KT} + C$$

Table II gives the observed values of the percentage denaturation and the values calculated from van't Hoff's equation, assuming the heat of reaction to be −67,600 calories per mole.

The Effect of Alcohol.—In general denaturing agents shift the equilibrium between native and denatured trypsin towards the denatured form. 10 per cent alcohol in 0.01 N hydrochloric acid lowers the temperature of half inactivation 5.5° (*cf.* Table I).

We are now in a position to understand why trypsin is such favorable material for the study of denaturation and its reversal. The first condition for the reversal of denaturation is that the denaturation procedure used should not cause secondary irreversible changes.

TABLE II

Effect of Temperature on the Equilibrium between Native and Denatured Trypsin in 0.01 N Hydrochloric Acid

Temperature	Percentage denaturation	Percentage denaturation calculated from $\ln K = -\frac{\Delta H}{KT} + C$ $-\Delta H = 67{,}600$ calories/mole
°C.		
42	32.8	32.8
43	39.2	41.0
44	50.0	50.0
45	57.4	56.4
48	80.4	80.0
50	87.8	87.2

Trypsin is remarkably stable in acid. The second condition for reversal is that the denatured protein be brought to a pH at which the native form is the equilibrium form. In the case of some proteins this means a pH close to the isoelectric point, *i.e.* a pH at which the denatured protein is insoluble, at which it is precipitated before reversal of denaturation can take place. Denatured trypsin changes into native trypsin in acid solutions in which denatured trypsin is entirely soluble.

EXPERIMENTAL

The trypsin used was prepared according to Northrop and Kunitz (1932). For the experiments of Table I the trypsin cake was simply diluted with the solvents described. For the experiments of Table II the trypsin was first dialyzed

in the cold. Dialysis does not affect the results because so much solvent is added to the trypsin cake that the salt introduced with the cake is eventually present in extremely dilute solution.

The method of estimating active native trypsin in the presence of inactive denatured trypsin by the use of a suitable hemoglobin solution has already been described (Anson and Mirsky, 1933).

The experiments with dilute trypsin in 0.01, 0.003, and 0.001 N hydrochloric acid, which include the experiments on the effect of temperature on the percentage denaturation, were carried out as follows: To estimate the activity before heating 5 ml. of hemoglobin solution plus 0.5 ml. water were poured into 0.5 ml. enzyme solution and the digestion was carried out for 5 minutes. Exactly the same procedure was used to measure the activity after heating and cooling. The heating consisted in keeping the test-tube containing the enzyme solution in a water bath 2° above the inactivation temperature for 2 minutes. The cooling consisted in keeping the trypsin solution at 25° for 10 minutes. The enzyme solution was heated to the inactivation temperature by being kept in a water bath at the inactivation temperature for 2–3 minutes and cooled to the inactivation temperature by being kept first in a bath 1–2° above the inactivation temperature for 1–2 minutes and then in a bath at the inactivation temperature for 2–3 minutes. The time at the higher temperature sufficed to produce more than half denaturation as was determined by separate experiments.

In the case of the ten times more concentrated trypsin in 0.01 N hydrochloric acid, the enzyme was first diluted ten times and a digestion mixture of the normal composition obtained by adding to 0.5 ml. of trypsin solution 4.5 ml. of a mixture of 10 parts hemoglobin solution and 0.8 parts water and then there were added to 1 ml. of the resulting solution 5 ml. of a mixture of 5 parts hemoglobin solution and 1 part water. The digestion time was measured from the first addition of hemoglobin.

In the case of the solution of trypsin in acid alcohol, the enzyme solution was kept at the inactivation temperature for 5 minutes and before being cooled to the inactivation temperature it was kept at a temperature 1.5° higher for 1 minute.

To estimate the activity in 0.05 N sodium hydroxide at 0°C. 1 ml. of 0.1 N sodium hydroxide was added to 1 ml. trypsin solution. After 1 minute there were added to 0.5 ml. of this solution a mixture of 5 ml. hemoglobin solution and 0.5 ml. 0.05 N hydrochloric acid. Digestion was carried out for 5 minutes. To find out how much of the trypsin was reversibly inactivated by the alkali, 14.5 ml. of 0.01 hydrochloric acid were added to 0.5 ml. of the alkaline trypsin and, after 10 minutes at 25°C., 5 ml. of hemoglobin solution were added to 1 ml. of the acidified trypsin and digestion was carried out for 5 minutes.

SUMMARY

There is a mobile equilibrium between the native and denatured forms of trypsin which depends on the concentrations of acid, alkali, and alcohol and on the temperature.

The heat of denaturation in 0.01 N hydrochloric acid calculated from the effect of temperature on the equilibrium constant is −67,600 calories per mole.

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THE EQUILIBRIA BETWEEN NATIVE AND DENATURED HEMOGLOBIN IN SALICYLATE SOLUTIONS AND THE THEORETICAL CONSEQUENCES OF THE EQUILIBRIUM BETWEEN NATIVE AND DENATURED PROTEIN

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The denaturation of hemoglobin by acid is partially reversible (Anson and Mirsky, 1931). If acidified hemoglobin is rapidly neutralized all the protein is precipitated. If the acidified hemoglobin is first made slightly alkaline and then after a few seconds brought to the neutral point only a third of the protein is precipitated. The soluble two-thirds has again the properties of native hemoglobin.

Equilibria in Salicylate Solutions.—It has already been shown (Anson and Mirsky, 1929 *b*) that concentrated sodium salicylate in neutral solution denatures hemoglobin and keeps denatured hemoglobin in solution. It will be shown in this paper that denaturation by salicylate is completely reversed when the salicylate is removed by dialysis under suitable conditions or when the salicylate solution is simply diluted with water. Salicylate not concentrated enough to denature hemoglobin completely produces an equilibrium mixture of native and denatured hemoglobin. The higher the salicylate concentration the higher is the percentage denaturation (see Fig. 1). At any given salicylate concentration the percentage denaturation is the same whether one starts with native or with denatured hemoglobin. Decreasing the hemoglobin concentration from 1 per cent to 0.5 per cent or raising the temperature from 25°C. to 35°C. has no detectable effect on the equilibrium in 0.25 M salicylate solution.

Differences between Native and Denatured Hemoglobin.—Hemoglobin denatured by salicylate has three properties characteristic of hemoglobin denatured by other means. It is insoluble under the same

conditions under which native hemoglobin is soluble; it is digested by trypsin which does not attack native hemoglobin; and it has the parahematin type of spectrum which is also given by a solution of hemin in pyridine. When the denaturation of hemoglobin by salicylate is reversed, the original properties of native hemoglobin are restored.

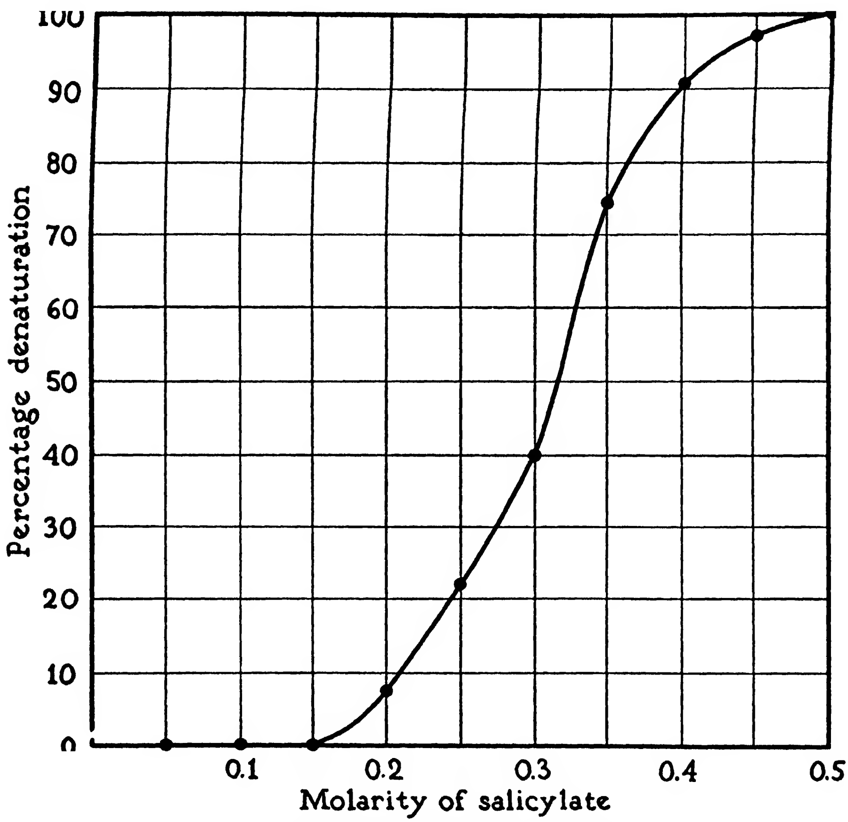

FIG. 1. The effect of salicylate concentration on the denaturation of hemoglobin

One can, if one so wishes, assume that salicylate converts hemoglobin not into denatured hemoglobin but into some other compound which also is insoluble, and digestible and has the parahematin spectrum. Before such an assumption need be considered seriously some difference between the hypothetical other compound and denatured hemoglobin must first be demonstrated.

The Change in Spectrum on Denaturation.—The change in spectrum resulting from the denaturation of hemoglobin is the basis of the method used to estimate the percentage denaturation. Hemoglobin consists of a native protein, globin, joined to an iron porphyrin complex, heme. The spectrum of heme is changed by combination of heme with native globin and the spectrum of the heme-globin compound is changed when the globin is denatured. The denaturation of hemoglobin can, therefore, be followed spectroscopically, heme acting as an indicator of the change in the protein with which it is combined. This was first pointed out for the compounds of reduced (ferrous) heme. The compound of reduced heme and native globin has the spectrum of reduced hemoglobin while the compound of reduced heme with denatured globin has the spectrum of hemochromogen (Anson and Mirsky, 1925; 1928). Analogously the compound of oxidized heme and native globin has the spectrum of methemoglobin while the compound of oxidized heme and denatured globin has the spectrum of parahematin (Keilin, 1926). Parahematin has no distinct absorption in either the yellow or the red, whereas alkaline methemoglobin and hematin have a band in the yellow and acid methemoglobin and hematin have a band in the red. The spectrum of parahematin is thus qualitatively different from the spectra of the other hemoglobin derivatives.

Because of technical difficulties the spectroscopic study of the denaturation of hemoglobin and its reversal has not been satisfactory. The difficulties are these. In neutral solution both globin hemochromogen and globin parahematin are insoluble. In alkaline solution, globin hemochromogen prepared from hemoglobin can combine with extra reduced heme and globin parahematin dissociates to a greater or lesser extent into denatured globin and oxidized heme. It is doubtful whether the pure spectrum of globin parahematin has hitherto been observed. In the neutral salicylate solutions used in the present experiments globin parahematin is neither precipitated nor dissociated and so the difficulties which have been mentioned are avoided.

The Change in Absorption of Green Light on Denaturation.—Viewed in the monochromatic green light easily obtained by the use of color filters from the mercury arc lamp, all pigments appear to be of the same color. Different pigments, however, differ in the extents to

which they absorb the green light. Denatured methemoglobin prepared by the addition of salicylate to a neutral phosphate solution of native methemoglobin absorbs the green light about twice as strongly as does native methemoglobin. Hence one can measure the extent of denaturation of methemoglobin by measuring the extent of absorption of green light with a colorimeter. The greater the percentage denaturation, the greater the absorption of green light.

Aggregation.—The solubility of denatured hemoglobin in dilute neutral salicylate solution is limited. If the protein concentration is too high there results, first, association or aggregation of the protein molecules, and then visible precipitation. Aggregation of the molecules of denatured hemoglobin increases the absorption of green light by an equilibrium mixture of native and denatured hemoglobin in two different ways. First, the aggregated pigment has a greater absorbing power than the unaggregated. Secondly, if as a result of aggregation denatured hemoglobin is removed from the equilibrium mixture

$$\text{unaggregated native hemoglobin} \leftrightarrows \text{unaggregated denatured hemoglobin}$$

then more denatured hemoglobin is formed to maintain the equilibrium and there is an increase in the total amount of denatured protein and hence in the light absorption. This complicating effect of aggregation on the study of an equilibrium has already been discussed in connection with the equilibrium

$$\text{reduced heme} + \text{pyridine} \leftrightarrows \text{pyridine hemochromogen}$$

(Anson and Mirsky, 1929*a*; 1930). The same formation of denatured from native hemoglobin which takes place when the denatured hemoglobin is aggregated also takes place when the denatured hemoglobin is digested.

To avoid aggregation in experiments on the effect of salicylate on the equilibrium between native and denatured hemoglobin, the hemoglobin concentration is kept as low as is consistent with accurate colorimetric measurements. Bovine hemoglobin is used because compared with hemoglobin from other common animals it is relatively soluble and requires a relatively high concentration of salicylate for denaturation. The solutions cannot be made more alkaline to avoid

aggregation because the optical properties of the pigments change and become much more alike than they are in neutral solution.

If salicylate is added to native methemoglobin the absorption of green light at first increases rapidly with time, then remains constant, and finally increases again very slowly. If the same final conditions are obtained by the addition of water to denatured methemoglobin in

TABLE I

Effect of Salicylate Concentration and Time on the Absorption of Green Light by Hemoglobin Solutions

Molarity of salicylate	Time, *min.*							
	0.5	5	10	15	30	40	60	120
0	20				20			
0.05 (*a*)				20.2		20.1		
0.10 (*a*)				20.1		20.1		
0.15 (*a*)		20.0		20.0				
(*b*)		20.0		20.0				
0.20 (*a*)	20.0	19.7	19.2	19.1	18.7	18.7	18.0	17.5
(*b*)	18.0	18.7	18.7	18.7	18.7	18.3	17.4	17.4
0.25 (*a*)	18.0	17.3	16.9	16.8	16.7	16.7	16.0	16.0
(*b*)	15.7	16.7	16.7	16.7	16.7	16.0	16.0	16.0
0.30 (*a*)	17.0	15.1	14.7	14.7	14.6	14.5	14.0	13.8
(*b*)	12.3	14.2	14.7	14.7		14.6	13.9	13.9
0.35 (*a*)	13.5	12.3	11.8	12.0	12.0	12.0	11.8	11.8
(*b*)	11.3	12.0	12.0	12.0	11.9	12.0	11.8	11.8
0.40 (*a*)	13.0	11.2	11.0	11.0				
(*b*)	10.8	11.0		11.0				
0.45 (*a*)	13.0	11.0	10.7	10.7				
0.50 (*a*)		10.5			10.5			

The figures represent colorimeter readings. (*a*) means that the equilibrium was reached by the formation of denatured hemoglobin, (*b*) by the formation of native hemoglobin. (See experimental part of text.)

concentrated salicylate solution then the light absorption first decreases rapidly, then remains constant, and finally increases very slowly. The value of the light absorption which is constant for a while is the same whether the experiment is started with native or with denatured hemoglobin. These results, which are given in Table I, suggest that a true equilibrium is measured before slow aggregation or other change takes place. Aggregation, however, is not definitely

excluded at any stage of the reaction. The state of dispersion of the protein can be decided conclusively only by direct molecular weight determinations.

That there is an equilibrium between a red form and a brown form of hemoglobin is an observed fact whose validity does not depend on the existence or non-existence of aggregation. If aggregation does take place before the equilibrium can be reached and measured then one cannot tell from the total effects of salicylate concentration and temperature on the percentage denaturation to what extent the salicylate concentration and the temperature influence the degree of aggregation and to what extent they influence the equilibrium between unaggregated native and denatured hemoglobins. If, on the other hand, the equilibrium is not being disturbed by aggregation then any theory of denaturation must be in harmony with the facts first, that the curve relating percentage denaturation to salicylate concentration is S-shaped (see Fig. 1) and, secondly, that temperature has little effect on the equilibrium between native and denatured hemoglobin although it has a great effect on the equilibrium between native and denatured trypsin (Anson and Mirsky, 1934). The theory of denaturation we shall now present which is in harmony with the facts which have just been stated is simply a restatement in other words of the existence of an equilibrium between the native form of a protein N and its denatured form, D.

Let us suppose that there is added to the equilibrium mixture $N \rightleftarrows D$, a substance, X, which can combine reversibly with both N and D or modify N and D in any reversible way. There then results the double equilibrium

$$\begin{array}{ccc} N + X & \leftrightarrows & NX \\ \downarrow\uparrow & & \uparrow\downarrow \\ D + X & \leftrightarrows & DX \end{array}$$

The total native protein is $N + NX$, the total denatured protein $D + DX$. There are two ways in which the equilibria can be influenced reversibly. First, the equilibrium constants can be changed by a change in the solvent, in the temperature, or in the rate at which any form of energy is being absorbed. Secondly, the amounts of N and D combined with X can be increased by an increase in the

concentration of X. If X has the same affinity for D as for N, then the fraction of D converted into DX by the addition of X is the same as the fraction of N converted into NX and there is no change in the total percentage denaturation. If X has a greater affinity for D than for N, relatively more D is converted into DX than N into NX and the percentage denaturation is increased. The necessary relation between the denaturation equilibria and the X combination equilibria is given by the identity:

$$\frac{N/D}{NX/DX} \equiv \frac{N/NX}{D/DX}$$

Given the mere fact that the addition of X causes denaturation one cannot decide whether the cause of the denaturation is a change in the equilibrium constants of the individual equilibria or a greater combination of X with D than with N, or whether X acts in both ways. The decision must be made on the basis of measurements of the individual equilibria or on the basis of chemical probabilities. It is likely, for instance, that a change in percentage denaturation brought about by acid is due to a difference in the affinities of acid for the native and denatured forms of the protein.

In practice when X is added to a protein there usually results a whole series of X compounds and hence the complicated equilibrium.

$$\begin{array}{ccccccc} N & \leftrightarrows & NX_1 & \leftrightarrows & NX_2 & \cdots\cdots & NX_n \\ \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \\ D & \leftrightarrows & DX_1 & \leftrightarrows & DX_2 & \cdots\cdots & DX_n \end{array}$$

The shape of the curve relating the concentration of X to the percentage denaturation depends on the values of all the equilibrium constants. By substituting a suitable set of values into the involved equation representing the complicated equilibrium one can obtain the S-shaped curve which relates the concentration of salicylate to the percentage denaturation of hemoglobin by salicylate. Such curve fitting is of little theoretical significance so long as the numerous equilibrium constants are chosen arbitrarily. What are needed are X combination curves to go with X denaturation curves; for instance, acid titration curves to go with measurements of the effect of pH on denaturation. The presentation of the detailed mathematical formu-

lation of the theory of equilibria had best be postponed until data for testing the equations are available.

The heat of the denaturation $N \rightarrow DX$ caused by the addition of X to N is equal to the heat of the reaction $N \rightarrow NX$ plus the heat of the reaction $NX \rightarrow DX$. Since the reaction $N \rightarrow NX$ may be either endothermic or exothermic depending on the nature of X, the heat of the denaturation $N \rightarrow DX$ will vary with the nature of the denaturing agent.

EXPERIMENTAL

Equilibria.—The stock solutions are a freshly filtered 1 per cent solution of dialyzed bovine methemoglobin prepared according to Anson and Mirsky (1931) in a buffer made up of equal parts 0.1 M K_2PO_4 and 0.1 M KH_2PO_4 and a filtered 1 M solution of sodium salicylate which is stored in the cold. Only such a sample of salicylate should be used which on filtration yields a water-clear solution. Monochromatic green light for the colorimetric measurements is obtained from the mercury vapor lamp by means of the two color filters supplied by the Corning Glass Company for the isolation of the green line. The standard which is set at 20 is made up by adding 9 parts of water to 1 part of the hemoglobin solution. All the experiments described in the experimental part are carried out at 25°C.

To reach the equilibrium corresponding to X tenth molar salicylate solution by formation of denatured hemoglobin, 10 ml. of solution are made by adding X ml. of the 1 M salicylate to a mixture of 1 ml. hemoglobin and $10 - (X + 1)$ ml. water. To reach equilibrium from the other side by the formation of native hemoglobin, 10 ml. of solution are made by adding X ml. of salicylate to a mixture of 1 ml. hemoglobin and $7 - (X + 1)$ ml. water and then after 3 minutes adding 3 ml. more of water. The colorimetric readings at various times are given in Table I.

Since denatured methemoglobin absorbs green light $\frac{20}{10.5}$ or 1.9 times as strongly as native methemoglobin, the relation between the colorimetric reading, R, and the fraction, D, of the protein which is denatured is given by

$$(1 - D) + 1.9D = \frac{20}{R}$$

or

$$D = \frac{20 - R}{0.9R}$$

The greater the percentage denaturation, the less accurate is the estimation of the percentage denaturation by this colorimetric method. Fig. 1 shows the relation between the salicylate concentration and the percentage denaturation.

The Digestion Test.—Hemoglobin in salicylate solution is not digested by trypsin unless the salicylate concentration is high enough to cause some denaturation as shown by the optical test. If a 1 per cent solution of hemoglobin in 0.3 M salicylate, which by the colorimetric test is about 40 per cent denatured and 60 per cent native, is diluted with equal volume of water and placed in boiling water the protein precipitates. If 1.7×10^{-8} hemoglobin units (Anson and Mirsky, 1933) of trypsin are added to each ml. of the solution before it is diluted and heated, then digestion of the denatured hemoglobin takes place, more denatured hemoglobin is formed to maintain the equilibrium and so on until after 20 minutes no precipitate is formed if the solution is diluted and heated. A 1 per cent solution of hemoglobin in 0.1 M salicylate which by the colorimetric test is all native even after 20 minutes treatment with trypsin yields the same sort of precipitate on dilution and heating as does hemoglobin not treated with trypsin. The dilution is made with 0.2 M salicylate instead of with water so that the final salicylate concentrations of the solutions which are heated are 0.15 M in the two cases. Native hemoglobin prepared from denatured hemoglobin behaves like native hemoglobin which was never denatured. To denature the hemoglobin 1 ml. of salicylate is added to 1 ml. of hemoglobin. Brown native hemoglobin is obtained again from the red denatured hemoglobin by the gradual addition of 7.5 ml. water. Finally 0.5 ml. trypsin solution is added. Since trypsin is probably destroyed under the conditions under which hemoglobin is inactivated there is probably more active trypsin in the solution of native hemoglobin which is not digested than in the solution of denatured hemoglobin which is digested.

The Solubility Test.—Denatured methemoglobin is insoluble in 0.3 saturated ammonium sulfate. 1 ml. salicylate is added to 1 ml. hemoglobin and the protein is then precipitated by the addition of a mixture of 12 ml. water and 6 ml. saturated ammonium sulfate. Native hemoglobin is soluble under the same final conditions. No

precipitate results from the addition of 6 ml. saturated ammonium sulfate to a solution prepared by adding 1 ml. hemoglobin to a mixture of 12 ml. water and 1 ml. salicylate. "Reversed" hemoglobin behaves like native hemoglobin. 1 ml. salicylate is added to 1 ml. hemoglobin to bring about denaturation; 12 ml. are then added gradually to bring about the reversal of denaturation. When finally 6 ml. of saturated ammonium sulfate are added only a slight haze is obtained. The clear brown filtrate from this solution stays clear.

SUMMARY

The denaturation of hemoglobin by salicylate in neutral solution is completely reversible.

There is a mobile equilibrium between native and denatured hemoglobin in neutral salicylate solution. The higher the salicylate concentration the greater is the percentage denaturation.

When there is a mobile equilibrium between the native and denatured forms of a protein, denaturation is caused by the addition of any substance which has a greater affinity for the denatured than for the native form.

Theoretically the heat of denaturation must vary with the denaturing agent and must depend on the heat of combination of the denaturing agent with the protein.

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ABSORPTION OF PEPSIN BY CRYSTALLINE PROTEINS

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Pepsin, like other enzymes, is removed more or less completely from solution by various insoluble substances. Dauwe (1) showed that insoluble proteins were particularly efficient in this respect. These results were confirmed by Abderhalden and coworkers (2). The writer found (3) that the quantity of pepsin removed by insoluble proteins depended largely upon the pH of the solution and that under certain conditions the ratio of the enzyme in the precipitate to that in the solution was the same as the chloride ion ratio. This result suggested that the pepsin was a negative ion and was distributed like any other ion in accordance with the Donnan equilibrium (4). In the acid range, however, between pH 2.0 and 5.0 the results were anomalous from this point of view since much more pepsin was absorbed than would be expected from the Donnan equilibrium.

It has recently been found by Dyckerhoff and Tewes (5) and by Waldschmidt-Leitz and Kofrányi (6) that crystalline proteins such as edestin or melon globulin also possess the property of absorbing pepsin from pepsin solutions, and Waldschmidt-Leitz considers that the crystalline foreign protein removes the active group from the pepsin protein. If this explanation were correct a convenient means would be at hand to separate the active group of pepsin from the protein-pepsin molecule, since the foreign protein (edestin or melon globulin) is rapidly and completely digested by pepsin and since there is little or no loss in activity during peptic digestion. It would only be necessary, therefore, to allow the complex of foreign protein and pepsin to digest until all the protein had been destroyed and the active pepsin must then be found in solution free from protein. When this experiment is performed, however, it is found that there is left in the digested

edestin solution an amount of pepsin protein just equivalent to the peptic activity present and equivalent to the loss in pepsin protein from the original pepsin solution. The absorption of pepsin by crystalline foreign protein, therefore, consists in the absorption of the pepsin protein, as such, and does not separate the pepsin protein into an inert protein and an active pepsin group.

The absorption of pepsin by edestin shows a sharp maximum at about pH 4.0 and pepsin may be removed completely from dilute solutions by stirring with a suspension of edestin crystals at this pH. The pepsin protein may be recovered from the "edestin-pepsin" complex by allowing the "edestin-pepsin" to autolyze, or by simply extracting the edestin-pepsin with N/4 sulfuric acid at 0°C. The recovered pepsin may be identified by its tyrosine-tryptophane content which is twice that of edestin and by its content of basic nitrogen which is about one-quarter that of edestin. It may be readily recrystallized and obtained in the characteristic crystalline form and with the characteristic specific activity of the original crystalline pepsin. If a suspension of edestin crystals at pH 4.0 is added to increasingly concentrated solutions of either crystalline or crude pepsin surprisingly large amounts of pepsin are taken up by the edestin crystals and preparations may be obtained which contain nearly 50 per cent pepsin and are, therefore, one-half as active as crystalline pepsin itself and much more active than commercial pepsin preparations. The general form and appearance of the edestin crystals is not markedly changed, but if the suspension of edestin crystals in the pepsin solution is allowed to stand for several hours at room temperature the edestin gradually dissolves and the pepsin content of the remaining precipitate increases. On longer standing the precipitate becomes less and less in bulk and finally dissolves completely, so that the final result is a solution of digested edestin containing the original quantity of pepsin.

The rate of autolysis can be increased by dissolving the edestin-pepsin precipitate in hydrochloric acid. The edestin protein is then very rapidly destroyed and there is left the pepsin protein. There is no change in activity during this process so that the autolysis of "edestin-pepsin" differs strikingly from the autolysis of pepsin itself, since in the latter case the destruction of the protein is paralleled by a

corresponding loss in activity while in the case of "edestin-pepsin" the edestin is destroyed without any corresponding loss in activity. There is, therefore, no reason to consider the "edestin-pepsin" complex as having any activity of its own aside from that due to the content of pepsin protein.

If supersaturated solutions of autolyzed edestin-pepsin or autolyzed solutions of pepsin alone are allowed to stand, the pepsin precipitates out in the form of spheroids, as Dyckerhoff and Tewes have shown (5). It is characteristic of proteins to appear in this spheroidal form when conditions are not quite right for crystallization or when they have not been sufficiently purified. The pepsin spheroids consist largely of pepsin but contain from 10 to 30 per cent non-protein nitrogen. They may be purified by solution and precipitation with acid or magnesium sulfate and the pepsin may then be obtained in the usual crystalline form.

The edestin-pepsin complex may also be formed by mixing cold solutions of edestin with solutions of pepsin. A precipitate forms which varies in composition and quantity with the pH of the solution. The maximum quantity and the maximum activity again are found at about pH 4.0. If the relative concentrations of pepsin and edestin are varied at pH 4.0 the quantity of pepsin in the precipitate is a maximum when equal concentrations (by weight) of pepsin and edestin are mixed. Under these conditions the precipitate contains nearly 75 per cent pepsin and is about three-quarters as active as crystalline pepsin itself.

Since pepsin and edestin both have an equivalent weight of about 1,000 the precipitate having maximum activity corresponds approximately to three equivalents of pepsin to one of edestin. Since the molecular weight of pepsin is only one-sixth that of edestin this corresponds approximately to eighteen molecules of pepsin to one of edestin.

Similar experiments may be performed with the globulin from melon seed (*Cucumis*), as Waldschmidt-Leitz and Kofrányi (6) have found, and also with gelatin. In both cases the pepsin protein removed from the pepsin solution and taken up by the solid protein corresponds to the loss of activity of the solution.

EXPERIMENTAL RESULTS

I. Absorption of Pepsin by Edestin Crystals from Crystalline Pepsin Solutions at Various pH

Experimental Procedure.—0.1 per cent solution of crystalline pepsin was titrated to various pH with sulfuric acid, cooled to 0°C. and 1 gm. crystalline edestin[1] (La Roche) was then added to 25 ml. of the solutions. The suspensions were stirred for a few minutes and kept at 6°C. for 18 hours. The suspensions were then centrifuged, the precipitates washed with 10 ml. of water and dissolved in 25 ml. of N/20 hydrochloric acid. Samples of the original suspensions, the supernatant solutions and the solutions of the precipitates were then analyzed for pepsin nitrogen, total nitrogen, tyrosine equivalent and peptic activity by the hemoglobin method (7). Pepsin nitrogen was determined by titrating the samples to pH 2.0 with hydrochloric acid and keeping at 33°C. for 3 hours. Any foreign protein is digested under these conditions and the protein nitrogen remaining is determined as usual by precipitation with hot trichloracetic acid. This protein nitrogen is called pepsin nitrogen. It is essential that hot trichloracetic acid be added to the cold pepsin solution as otherwise the pepsin may autolyze while the solution is being heated.

The tyrosine equivalent is determined by the development of the blue color with Folin's reagent; 3 ml. of the solution is treated with the reagent and the measurement carried out exactly as for the hemoglobin filtrate in the pepsin determination with hemoglobin. This gives the number of milligrams of tyrosine which would give the same color as the tyrosine plus tryptophane contained in the unknown solution.

The results of the analyses have been calculated to the basis of 1 ml. of the original suspension.

The results of the experiment are shown in Table I and Fig. 1. There is a sharp maximum of absorption at about pH 4.0 and in this range 90 per cent of the activity is found in the precipitate. Correspondingly about 90 per cent of the pepsin nitrogen is also in the precipitate. The pepsin nitrogen is identified by the fact that it is not digested if allowed to stand in acid solution and by its content of tyrosine plus tryptophane (tyrosine equivalent) of about 0.64 mg. tyrosine per mg. pepsin nitrogen, while the tyrosine equivalent of edestin is about 0.34. In this experiment, owing to the low concentration of pepsin the specific activity of the precipitate is low and is only about 3 or 4 per cent that of the crystalline pepsin.

[1] 1 gm. of this preparation contained 0.65 gm. dry edestin, as calculated from the nitrogen content.

TABLE I

Absorption of Pepsin by Edestin from Crystalline Pepsin Solutions at Various pH

pH		1.12	1.6	2.8	3.63	4.25	4.85	5.5	5.6	5.9	Control no edestin 4.5	Control no pepsin 5.9
Concentration H_2SO_4 — N		0.2	0.10	0.05	0.025	0.012	0.006	0.003	0.0015	0	0	0
$[P. U.]^{Hb}_{ml.}$	Suspension	0.028	0.027	0.027	0.026	0.028	0.028	0.027	0.026	0.027	0.028	0
	Supernatant	0.025	0.025	0.011	0.0006	0.0026	0.010	0.018	0.020	0.021	0.028	0
	Precipitate	0.0016	0.002	0.016	0.025	0.025	0.014	0.008	0.006	0.005	0	0
Per cent activity in precipitate		6	7.4	59	93	93	52	29	22	18	0	
Pepsin N/ml., *mg.*	Supernatant	0.14		0.056	<0.01	0.017	0.056	0.084	0.110		0.17	
	Precipitate	0.02		0.10	0.15	0.14	0.09	0.06	0.04			
Per cent total pepsin N in precipitate		12		63	94	87	56	37	25			
$[P. U.]^{Hb}_{mg.\ pepsin\ N}$	Supernatant	0.18		0.20		0.15	0.18	0.21	0.18		0.17	
	Precipitate	0.08		0.16	0.17	0.18	0.16	0.13	0.15			
Tyrosine equivalent per mg. pepsin N	Supernatant	0.56		0.54			0.54	0.64	0.64		0.60	0.34
	Precipitate				0.58	0.60						
Total N/ml., *mg.*	Supernatant	4.3		3.1	1.36	0.80	0.52	0.41	0.40	0.38	0.20	
	Precipitate (by difference)	0.7		1.9	3.6	4.2	4.5	4.6	4.6	4.6		4.8
$[P. U.]_{mg.\ N}$	Supernatant	0.006		0.004	0.0004	0.003	0.02	0.045	0.05	0.05	0.14	0
	Precipitate	0.002		0.0085	0.007	0.006	0.003	0.0017	0.0012	0.001		

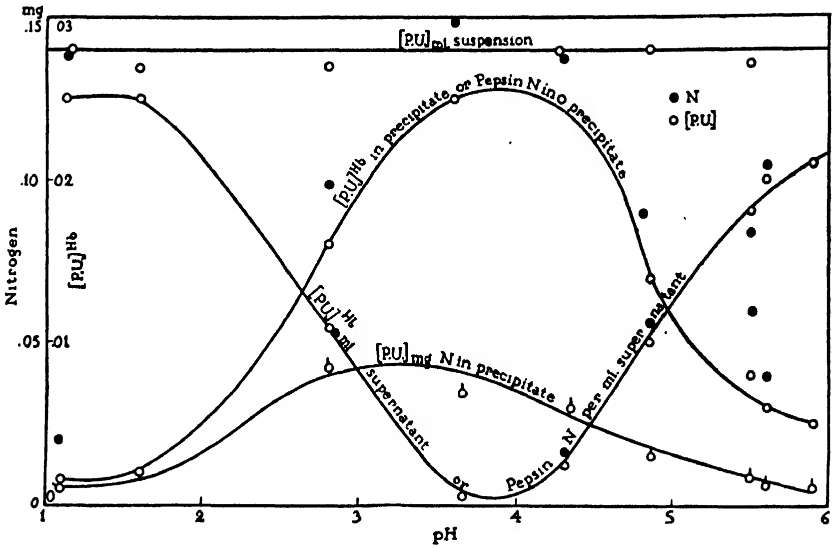

FIG. 1. Absorption of pepsin by crystalline edestin at various pH

II. Recovery of Crystalline Pepsin from Edestin-Pepsin Complex

Experimental Procedure.—

	Fraction No.
1000 gm. of Parke, Davis pepsin dissolved in 2500 ml. of water, pH about 5.0....	1
The solution cooled to 6°C. and 40 gm. crystalline edestin (La Roche) added, the suspension stirred for about 2 hours, filtered through fluted paper and the precipitate washed twice with water. Precipitate consisted of slightly deformed edestin crystals. The precipitate was suspended in water and titrated with hydrochloric acid to pH 2.5 and kept at 20°C. for 24 hours.....	2
A slight flocculent precipitate formed which was filtered off and suspended in N/50 hydrochloric acid..	3
Filtrate allowed to stand at 6°C. for 3 weeks. Dark, oily gum settled on the bottom; insoluble in alkali or acid and contained very little activity. Supernatant solution decanted from this gum and the protein precipitated by the addition of 1 volume of saturated magnesium sulfate and filtered. The precipitate was suspended in 8 ml. of water, warmed to 45°C. and dissolved by the addition of a few drops of N/10 sodium acetate...........	7
N/2 sulfuric acid added until slight cloud appeared and the solution allowed to cool slowly. Precipitate consisted of spheroids mixed with some amorphous material and a few pepsin crystals............................	8

Precipitate redissolved at 45°C. and recrystallized as above. Precipitate consists of pepsin crystals; dissolved in pH 5.0 sodium acetate.......... 11
Filtrate.. 10
This filtrate was allowed to stand at 6°C. and after 2 weeks most of the pepsin had crystallized out

The various fractions obtained in this way were analyzed for total nitrogen, pepsin nitrogen, tyrosine equivalent, and basic nitrogen and their activities determined by a series of methods (2). Basic nitrogen is determined by dissolving the sample in 5 M hydrochloric acid and heating in the autoclave at 120°C. for 2 hours. Total nitrogen is then determined on 1 ml. of this solution; 2 ml. of solution is added to 2 ml. of a saturated solution of phosphotungstic acid in 5 M hydrochloric acid and the mixture left 18 hours at 0°C. It is centrifuged and the total nitrogen determined on 2 ml. of the supernatant solution. The difference between this figure and the total nitrogen is the basic nitrogen.

The results are tabulated in Table II and are expressed as the total nitrogen or activity present in the entire fraction. The results show that in this case about 1 per cent of the total activity and also of the pepsin nitrogen was taken up by the edestin. The edestin-pepsin had about one-half the activity of the original Parke, Davis pepsin on the basis of total nitrogen content, as determined by any of the methods used, except the gelatin viscosity method. The activity by the gelatin viscosity method is the same as that of the Parke, Davis pepsin so that the edestin has a slightly preferential affinity for the gelatinase fraction. After autolysis and precipitation with magnesium sulfate most of the activity is found in the precipitate which now has about one-half the specific activity, on a total nitrogen basis, of the crystalline pepsin. This precipitate of amorphous pepsin when dissolved with alkali and then acidified and allowed to cool appears in the form of spheroids ("*kugeln*") mixed with a few crystals which have about the same specific activity as the amorphous precipitate. When these spheroids are dissolved and crystallized in the usual way normal pepsin crystals are obtained with the same specific activity as the ordinary crystalline pepsin except that they contain considerable gelatinase, as shown by higher specific activity as measured by the viscosity of gelatin. Once crystallized pepsin always contains more or less of this gelatinase fraction and four or five crystallizations are required to free the pepsin completely from the gelatin-splitting enzyme (9).

TABLE II

Analysis of Various Fractions

	Method	P. D. pepsin	"Edestin-pepsin"	Amorphous pepsin	Spheroids	Filtrate	Pepsin crystals	5 × crystalline pepsin	Edestin
Fraction No.		1	2	7	8	10	11	5/9/32	
Total N, *mg.*		200,000	5000	116	35	10	11		
Total pepsin N, *mg.*		20,000	210	84	28	5	10		
$[\text{P. U.}]^{\text{Hb}}$ total		3000	35	12	4.2	0.7	1.6		
$[\text{P. U.}]_{\text{mg. N}}$	Hb.	0.015	0.007	0.10	0.12	0.07	0.15	0.20	
	Gel. V^-	2	2	12	12.7	6.2	28.5	11.0	
	Cas. V^-	150	66	1000	1100	700	1450	1700	
	Ed. V^-	170	72	1000	950	786	1250	1650	
	Cas. S.	0.05	0.02	0.36	0.33	0.27	0.5	0.5	
	Cas. V^+	30	13	180	160	160	200	310	
	Ren.	4×10^4	1.6×10^4	50×10^4	30×10^4	20×10^4	60×10^4	44×10^4	
$[\text{P. U.}]_{\text{mg. pepsin N}}$	Hb.	0.15	0.16	0.15	0.15	0.14	0.16	0.20	
	Gel. V^-	18	40	16	17	12	31	11	
	Cas. V^-	1400	1500	1450	1500	1500	1500	1700	
	Ed. V^-	1500	1500	1320	1300	1400	1360	1650	
	Cas. S.	0.47	0.5	0.5	0.46	0.54	0.54	0.5	
	Cas. V^+	280	290	250	230	300	220	310	
	Ren.	36×10^4	38×10^4	60×10^4	40×10^4	40×10^4	50×10^4	44×10^4	
Tyrosine equivalent per mg. pepsin N		0.5		0.48	0.50	0.56	0.56	0.67	0.32
Basic N as per cent pepsin N		8	7.5				6.7	8	35

When the specific activity is calculated on the basis of the pepsin nitrogen content, *i.e.* protein nitrogen which is not destroyed upon standing in acid solution, the specific activity remains constant throughout all fractionations, as shown by the lower part of the table.[2] In other words, the content of pepsin protein present in every fraction is just sufficient to account for the observed activity. The fact that this protein nitrogen is really pepsin nitrogen and not edestin nor one of its decomposition products is shown by the fact that the tyrosine equivalent and the basic nitrogen content is that of pepsin and not of edestin. Identification is made complete by the actual recovery of typical pepsin crystals. The per cent actually recovered in crystalline form is rather small but is about what would be expected from a

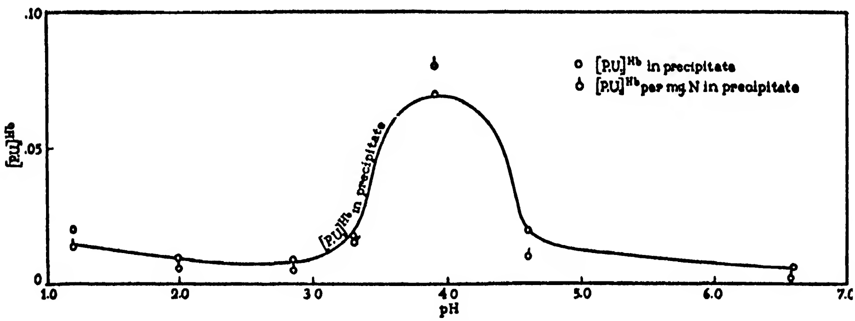

FIG. 2. Absorption of pepsin by crystalline edestin from solutions of Cudahy pepsin at various pH.

solution containing such a large amount of protein decomposition products.

The method of determining activity by formol titration of Dyckerhoff and Tewes was not used in general since it is very troublesome and inaccurate but the

[2] It may be noted that the specific activity of this sample of Parke, Davis pepsin was only about one-half that usually found and also that the tyrosine equivalent per mg. nitrogen is slightly low. Both these results are due to the presence of an inert protein in this particular sample which is evidently carried through the adsorption procedure. This inert protein may be removed by allowing the solutions to stand longer in acid solution, but since there is a slight loss of activity under this condition this procedure could not be used for analytical purposes. The activity by some methods, especially edestin viscosity, differs from that previously obtained (8) owing to variations in the edestin solution.

results with this method agree approximately with those given by Dyckerhoff and Tewes, as shown in Table III. The casein solution used was made up as described by Dyckerhoff and Tewes (5) for their "pH 4.0 casein." However, the pH of this solution, as determined by the hydrogen electrode or quinhydrone electrode is about 2.35, although methyl-orange gives an apparent pH of about 4.0. Dyckerhoff and Tewes used the indicator method[3] for determining pH and their pH values are from 1 to 1.5 pH more alkaline than would be found by the hydrogen electrode method. This probably accounts also for the very alkaline optimum pH of digestion reported by these workers. Under the conditions used by Dyckerhoff and Tewes the casein is digested practically instantly, owing to the enormous concentration of pepsin added and the titration obtained represents the final stages of the digestion. Under these conditions the change in titration with increasing pepsin concentration is very slight so that there is a very large error. On the other hand, if the first part of the curve is used the increase in titration is extremely small and difficult to determine. The large quantity of enzyme used renders it difficult to obtain the initial titration since the reaction proceeds extremely rapidly at first and also renders it difficult to be sure that there is no change in pH of the casein solution caused by the addition of the pepsin. A number of very erratic results were obtained at first and were traced to marked changes in pH in the casein solution upon the addition of the pepsin. The table shows, however, that the activity of the various preparations agrees quite closely with that reported by Dyckerhoff and Tewes.

It will be noted that 24 mg. of enzyme gives only about 0.2 ml. more titration in 24 hours than 6 mg. of enzyme, *i.e.* a difference of 400 per cent in the pepsin concentration makes a difference in titration of only 0.2 ml. so that since the error in the titration is about ± 0.05 ml. there is an uncertainty of nearly 100 per cent in the method. The preparation of Cudahy pepsin used by Dyckerhoff and Tewes was apparently slightly more active than that used in the present experiments.

III. Extraction of Pepsin from "Edestin-Pepsin" with Sulfuric Acid

If edestin-pepsin prepared by absorbing pepsin with crystalline edestin is stirred at 0°C. with sulfuric acid at pH about 1.0, the pepsin dissolves out leaving inactive edestin crystals. The results of such an experiment are shown in Table IV. A preparation of "edestin-pepsin" of relatively low activity was used purposely since it would be expected that a small amount of pepsin would be removed with more difficulty than a larger amount. The experiment shows that 80 per cent of the activity and of the pepsin nitrogen is removed from the

[3] Personal communication from Drs. Dyckerhoff and Tewes.

TABLE III

Hydrolysis of Casein by Various Pepsin Preparations

8 ml. casein ("pH 4.0" Dykerhoff and Tewes) + 2 ml. pepsin solution at 35°C. Formol titration on 2 ml.

Enzyme preparation	Crystalline pepsin		Cudahy U.S.P. 1:10,000		"Edestin-pepsin"		
Mg. enzyme/2 ml. enzyme solution....	2.0	2.0	9.0	9.0	24	6	6
$[P.U.]^{Hb}_{ml.\ enzyme\ solution}$............	0.030		0.030		0.030		
	J.H.N.	D. and T., Table 3	J.H.N.	D. and T., Table 4	J.H.N.	J.H.N.	D. and T., Table 12a, Table 1a
Δ time	Δ ml. N/20 sodium hydroxide per 2 ml.						
hrs.							
1	0.26		0.22	0.29	0.26	0.16	
3				0.39			0.18
4	0.36		0.33		0.34	0.23	
6		0.35		0.43			
24	0.52	0.50	0.52	0.73	0.56	0.32	0.35

"edestin-pepsin" by the first extraction with sulfuric acid. The three following extractions remove practically all the remaining activity and pepsin nitrogen so that finally a little more than 90 per cent of the total original activity is recovered in the washings. The concentration of pepsin nitrogen in the second, third and fourth washings and in the final solid are too small to be accurately determined owing

TABLE IV

Extraction of Pepsin from Edestin-Pepsin

	Total N/ml.	Pepsin N/ml	$[P.U]^{Hb}_{ml.}$	$[P.U.]^{Hb}_{mg.N}$	$[P.U.]^{Hb}_{mg.\ pepsin\ N}$	pH
	mg.	*mg.*				
0.5 gm. "edestin-pepsin" + 10 ml. N/4 sulfuric acid.	10	0.30	0.05	0.005	0.17	0.8
Stir at 0°C. for 20 min.						
Centrifuge—supernatant	1.3	0.23	0.038	0.029	0.165	
Precipitate stirred + 10 ml. N/10 sulfuric acid.						
Centrifuge—supernatant	1.3	0.02	0.003	0.0025	0.15	
Precipitate stirred + 10 ml. N/10 sulfuric acid.						
Centrifuge—supernatant	1.4	0.02	0.0024	0 0017	0.12	
Precipitate stirred + 10 ml. N/10 sulfuric acid.						
Centrifuge—supernatant	1.2	0.02	0.0020	0.0016	0.10	
Precipitate + 10 ml. water—suspension edestin crystals	4 5	0.02	0.001	0.0002	0.05	
Total $[P.U.]^{Hb}$ in washings			0.454			
in precipitate			0.010			
			0.464			
Total original $[P.U.]^{Hb}$			0.50			

to the difficulty of completely digesting such a large excess of inert protein. Attempts to separate the complex by extraction or washing at pH 5.0 to 6.0, where pepsin itself is very soluble and edestin is insoluble, were not successful. If anything, the specific activity of the precipitate increases. Evidently the pepsin edestin complex is less soluble than edestin alone.

IV. Effect of the Concentration of Pepsin on the Absorption of Pepsin by Edestin at pH 4.0, 6°C. from Crystalline Pepsin Solutions

Experimental Procedure.—About 10 gm. of twice crystallized pepsin was stirred in 75 ml. of water and N/2 sodium hydroxide added until the solution was at pH 4.0 (clear solution). The solution was diluted with water to the concentrations noted in Table V, cooled to 6°C. and 20 ml. of the various dilutions added to a series of suspensions of 1 gm. of edestin in 5 ml. N/10 sulfuric acid in 250 ml. Erlenmeyer flasks; the suspensions were stirred occasionally and left at 6°C. for 18 hours. They were then centrifuged and the precipitates washed with 20 ml. cold water, dissolved in 25 ml. N/20 hydrochloric acid and allowed to stand at 24°C. for 24 hours. The solutions were then analyzed for peptic activity by the hemoglobin method, protein nitrogen, total nitrogen and tyrosine equivalent.

The results of the experiment are shown in Table V and Fig. 3. As the concentration of pepsin is increased the quantity of pepsin in the edestin crystals increases until a maximum value is reached which in this case corresponds to about 10 per cent pepsin. As usual, the pepsin protein taken up by the precipitate is just equivalent to the activity found in the precipitate. The pepsin protein has the characteristic tyrosine equivalent of pepsin and also the characteristic specific activity.[4]

V. Effect of the Pepsin Concentration on the Absorption of Pepsin by Crystalline Edestin from Solutions of Cudahy Pepsin at pH 4.0 at 6°C.

Experimental Procedure.—100 gm. Cudahy pepsin dissolved in 100 ml. water and the solution diluted with water to the concentrations noted and cooled to 6°C. 1 gm. of edestin in 5 ml. of N/10 sulfuric acid added to 20 ml. of the various pepsin solutions, stirred for 20 minutes and kept at 6°C. for 18 hours; pH of all solutions about 4.0. The supernatant was then centrifuged and the precipitate washed with 20 ml. water and dissolved in 25 ml. N/20 hydrochloric acid. The original suspension and the solution and precipitate were analyzed for total nitrogen, pepsin nitrogen and activity by the hemoglobin method.

The results have been calculated to the basis of 1 ml. of the original suspension and are given in Table VI and Fig. 3.

When Cudahy pepsin is used the activity of the precipitate increases to a maximum which, however, is considerably higher than was the case in the experiment with crystalline pepsin solutions. The pepsin

[4] See footnote, page 163.

TABLE V

Effect of the Concentration of Pepsin on the Absorption of Pepsin by Edestin at pH 4.0, 6°C. from Crystalline Pepsin Solutions

Suspension	Total N/ml, *mg.*	20 00	12.5	9 00	7.00	6.00	5.50	5 00	5.10	No pepsin	No edestin
	Pepsin N/ml., *mg.*	15.00	7.5	3.75	1.87	0.93	0 47	0 24	0 12		
	$[P.U.]^{Hb}_{ml.}$	2.60	1.3	0.65	0.33	0.17	0.085	0.048	0.020		
Precipitate	$[P.U.]_{ml.}$	0.065	0.071	0.070	0.080	0.078	0.049	0.024	0.012		
	Per cent total activity in precipitate	2.5	5.5	11.00	24.0	46.0	57.0	50.0	60.0		
	Pepsin N/ml., *mg.*	0.45	0.43	0.49	0.56	0.48	0.30	0.18	0.13		
	Total N/ml., *mg.*	4.7	5.1	5.2	5.0	5.2	5.1	5.0	5.1	4.8	
	$P.U.]^{Hb}_{mg.\ N}$	0.014	0.014	0.014	0.016	0.015	0.01	0.005	0.0025	.	0.15
	$[P.U.]^{Hb}_{mg.\ pepsin\ N}$	0.145	0.17	0.14	0.14	0.16	0.16	0.14	0.10		0.16
	Tyrosine equivalent/mg. pepsin N	0.60	0.60	0.60	0.57	0.58	0 60	0.60		0.34	0.60
	Per cent pepsin in precipitate	10.0	9.0	10.0	11.0	9.0	6.0	3.6	2.6		
	Mols pepsin/mols edestin	0.66	0.59	0.66	0.74	0 52	0 38	0 22	0.16		

nitrogen absorbed is again equivalent to the activity taken up by the precipitate. It will be noted that in this experiment the specific activity of the precipitate, calculated to the basis of pepsin nitrogen, is about 0.2 which is the usual value for crystalline pepsin, whereas in the preceding experiment it was only about 0.16. This is due to the fact that the crystalline pepsin used in the previous experiment was prepared from a commercial preparation which was peculiar in that it contained about 20 per cent of an inert protein very similar to pepsin

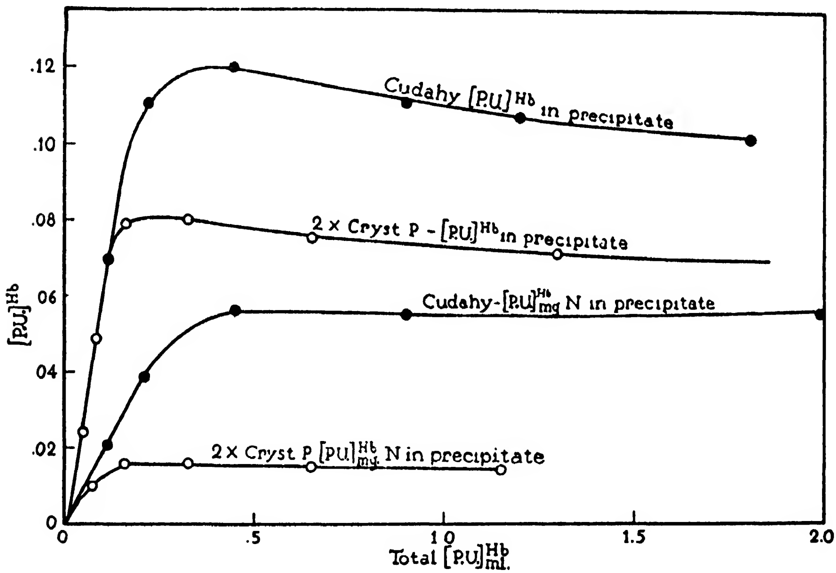

FIG. 3. Effect of the concentration of pepsin on the absorption of pepsin by edestin from solutions of crystalline pepsin or Cudahy pepsin.

itself and which could only be removed by five or more fractional crystallizations or by prolonged standing in acid solution. The preparation used in the experiment referred to had not been purified by fractional crystallization since it seemed possible that absorption by edestin might serve as a means of removing this inert protein. The results, however, show that this is not the case but that the inert protein is absorbed by the edestin to the same extent as the active enzyme so that the specific activity is not changed by the edestin treatment. The specific activity on a total nitrogen basis of the

TABLE VI

Effect of the Pepsin Concentration on the Absorption of Pepsin by Crystalline Edestin from Solution of Cudahy Pepsin at pH 4.0 at 6°C.

								No pepsin	No edestin
Suspension	Total N/ml., *mg.*	95	50	27	16	10.6	7.8	4.8	
	$[P.U.]^{Hb}_{ml.}$	3.6	1.8	0.90	0.45	0.22	0.11		
	Pepsin N/ml., *mg.*	18	9	4.5	2.2	1.1	0.55		
Precipitate	Protein N/ml., *mg.*	3.2	2	2	2.3	2.9	3.5	4.8	
	Pepsin N/ml., *mg.*	0.26	0.46	0.50	0.50	0.54	0.31		
	$[P.U.]^{Hb}_{ml.}$	0.056	0.10	0.11	0.12	0.11	0.068	0	
	Per cent total $[P.U.]^{Hb}$	1.5	5.5	12	27	50	62		
	$[P.U.]_{mg.\ protein\ N}$	0.017	0.05	0.055	0.052	0.038	0.02	0	0.04
	Per cent pepsin $\left(\frac{\text{Pepsin N}}{\text{Protein N}}\right)$	8.1	23	25	22	19	8.8		
	Mols pepsin/mols edestin	0.52	1.8	2.0	1.7	1.5	0.56		
	$[P.U.]^{Hb}_{mg.\ pepsin\ N}$	0.21	0.22	0.22	0.24	0.20	0.22	0	0.20

"edestin-pepsin" prepared with Cudahy solution is about one-third that of crystalline pepsin and is, therefore, nearly twice as high as that of the original Cudahy pepsin. This is near the maximum activity obtained in any experiment in which crystalline edestin was treated with pepsin solutions. It corresponds to about 30 per cent pepsin which is equivalent to about 3 moles of pepsin to 1 mole of edestin or about one-half an equivalent of pepsin per equivalent of edestin. The results of both experiments are shown in Fig. 3.

The low activity of the precipitate in the experiment with crystalline pepsin is due to some accidental condition, such as stirring or length of time in which the edestin was in contact with the sulfuric acid since in other experiments "edestin-pepsin" which had a much higher activity was prepared from crystalline pepsin solutions.

VI. Changes in Edestin-Pepsin Suspensions with Time

In the preceding experiments the edestin crystals were stirred with the pepsin solutions for a few minutes and then allowed to stand at 6°C. for 18 hours. In the present experiment the suspension was stirred for about 1 hour at 0°C. and then continued at 30°C. in order to accelerate the reaction so that it would be completed in a convenient time. The results of the experiment are shown in Fig. 4.

Experimental Procedure.—Pepsin solution, twice crystallized, pH 4.0. Edestin solution, crystalline (La Roche). 5 gm. edestin stirred with 20 ml. cold N/10 sulfuric acid and 80 ml. cold pepsin solution added. Suspension stirred at 0°C. for 1 hour and then at 35°C. 5 ml. samples centrifuged at intervals and precipitate dissolved in 5 ml. N/50 hydrochloric acid. Suspension, supernatant and precipitate analyzed for total nitrogen, total protein nitrogen, pepsin nitrogen and activity by hemoglobin method.

Total protein nitrogen: 1 ml. of solution plus 9 ml. boiling 5 per cent trichloracetic acid, cool, centrifuge, and wash precipitate with 5 per cent trichloracetic acid and nitrogen determined.

Pepsin nitrogen: 1 ml. plus 9 ml. N/20 hydrochloric acid, 37°C. for 3 hours. 5 ml. plus 5 ml. boiling 10 per cent trichloracetic acid and precipitate washed and analyzed for nitrogen.

Total protein nitrogen − pepsin nitrogen = edestin nitrogen.

Activity: Solution diluted to contain about 0.01 mg. pepsin N/ml. and activity determined by hemoglobin method.

All results calculated to the basis of 1 ml. of original suspension. Composition of original suspension calculated from analysis of pepsin and edestin alone.

	Total N	Pepsin N	Edestin N	$[\text{P U.}]^{\text{Hb}}$
	mg.	*mg.*	*mg*	
Pepsin	4.4	3.5		0.64
Edestin	7.6		7.6	

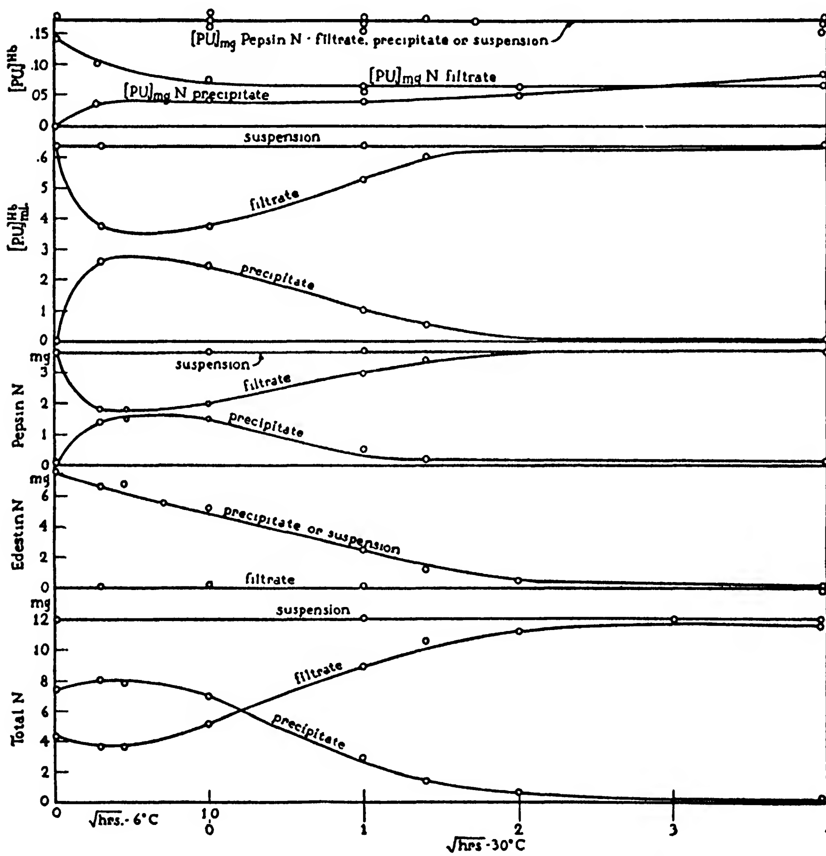

FIG. 4. Autolysis of edestin-pepsin suspensions with time.

In the first few minutes pepsin nitrogen and the corresponding amount of activity are taken up by the edestin crystals and there is a corresponding loss from the filtrate. As the reaction proceeds the

quantity of precipitate becomes gradually less, the loss being due to loss of edestin nitrogen. The pepsin nitrogen and activity in the precipitate decrease, and increase to a corresponding extent in the filtrate. The specific activity of the precipitate, however, continues to increase and reaches a value of about one-half that of crystalline pepsin at the end of 24 hours. At this time there is only a very small amount of precipitate left and the solution is practically a solution of pepsin containing digested edestin. It will be noted that there is practically no change in the total activity or total pepsin nitrogen of the whole suspension at any time.

VII. Changes in Protein Nitrogen and Peptic Activity during Autolysis of Edestin-Pepsin at pH 1.5 and 35°C.

In the preceding experiment the edestin-pepsin autolyzed at about pH 4.0. If the edestin-pepsin is dissolved in hydrochloric acid the edestin is destroyed much more rapidly.

Experimental Procedure.—2 gm. crystalline edestin suspended in 10 ml. of N/10 sulfuric acid, cooled to 0°C. and poured into 40 ml. of a solution of cold, twice crystallized pepsin pH 4.0 containing 15 mg. of pepsin nitrogen per ml. Suspension stirred for 20 minutes, centrifuged and the precipitate washed with 20 ml. cold water. The precipitate stirred with 20 ml. water and titrated to pH 1.5 with N hydrochloric acid; clear solution; total volume about 35 ml. This solution was placed at 35°C. and analyzed at intervals for total protein nitrogen, pepsin nitrogen and peptic activity by the hemoglobin method.

The results of such an experiment are shown in the lower part of Fig. 5. As in the preceding experiment there is no change in the total activity of the solution as a whole nor in the quantity of pepsin nitrogen present. The total protein nitrogen decreases rapidly and is practically reduced to the value of the pepsin nitrogen in 1 hour. The specific activity calculated on the basis of total protein nitrogen, therefore, increases rapidly and soon reaches the characteristic value for pepsin itself. In the upper part of the figure is shown the result of autolysis of a solution of pure pepsin. It is evident that the course of the reaction is entirely different. In this case the activity decreases in proportion to the total protein nitrogen (pepsin nitrogen) instead of remaining constant as is the case with "edestin-pepsin" preparations. As a result the specific activity calculated on a protein

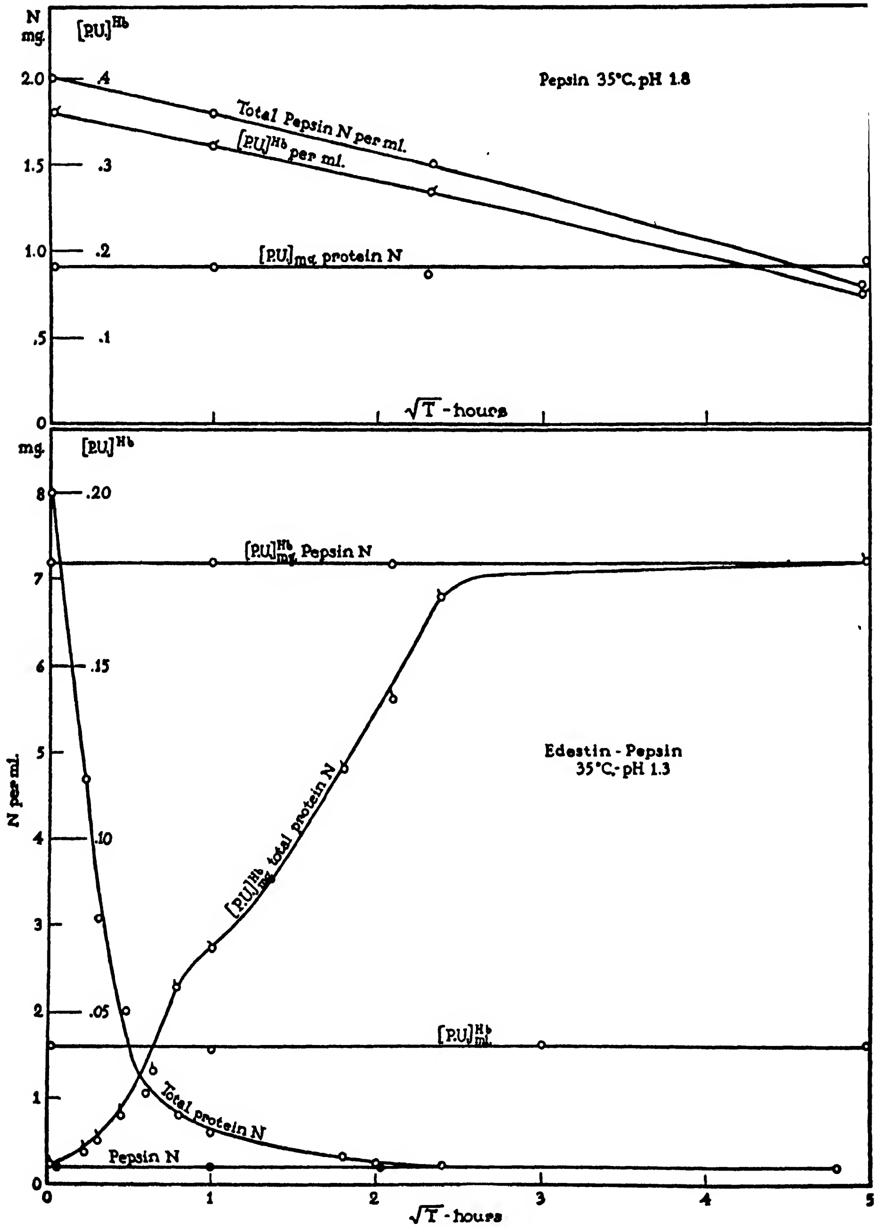

FIG. 5. Autolysis of pepsin (upper part) and of edestin-pepsin (lower part) in acid solution at 35°C.

nitrogen basis remains constant in the case of a solution of pepsin instead of increasing rapidly as in the case of a solution of "edestin-pepsin." Autolysis, therefore, serves to hydrolyze the edestin from the "edestin-pepsin" and leaves the pepsin. It will be noted that a solution of pure pepsin loses considerable activity whereas there is very little loss in activity from the "edestin-pepsin" solution. This is due to the protective effect of the products of digestion on the enzyme, probably through the formation of an additional compound of the enzyme with the products of hydrolysis of the edestin.

VIII. Preparation of Edestin-Pepsin from Edestin and Pepsin Solutions at Various pH

Experimental Procedure.—1 ml. of a 10 per cent solution of edestin in 5 per cent sodium chloride added to 10 ml. various concentrations hydrochloric acid and cooled to 0°C. 1 ml. of a solution of twice crystallized pepsin containing 1 mg. pepsin nitrogen per ml. added; precipitate appears. The suspensions kept at 0°C. for ½ hour, centrifuged and the precipitates washed once with 5 ml. cold water and dissolved in 10 ml. N/50 hydrochloric acid. The precipitate solutions were then analyzed for total nitrogen, pepsin nitrogen and peptic activity by the hemoglobin method. The edestin nitrogen is calculated as the difference between the total protein nitrogen and pepsin nitrogen. The results have been calculated to the basis of 1 ml. of the original suspension.

In the preceding experiments the edestin-pepsin was prepared by suspending edestin crystals in cold pepsin solutions. As shown by Dyckerhoff and Tewes, more active preparations may be prepared by mixing edestin solutions with pepsin solutions. The results of an experiment of this kind in which solutions were mixed at various pH are shown in Fig. 6. The figure shows that, as in the case of the absorption experiments, there is a sharp maximum at about pH 3.6. The quantity of pepsin nitrogen found in the precipitate is again equivalent to the activity of the precipitate and at the maximum amounts to 50 per cent of the precipitate. The specific activity of the precipitate at this point, therefore, is one half that of pepsin itself. As the pH becomes more and more alkaline the quantity of precipitate increases rapidly as does the per cent of edestin in the precipitate until beyond pH 5.0 the precipitate is practically all edestin.

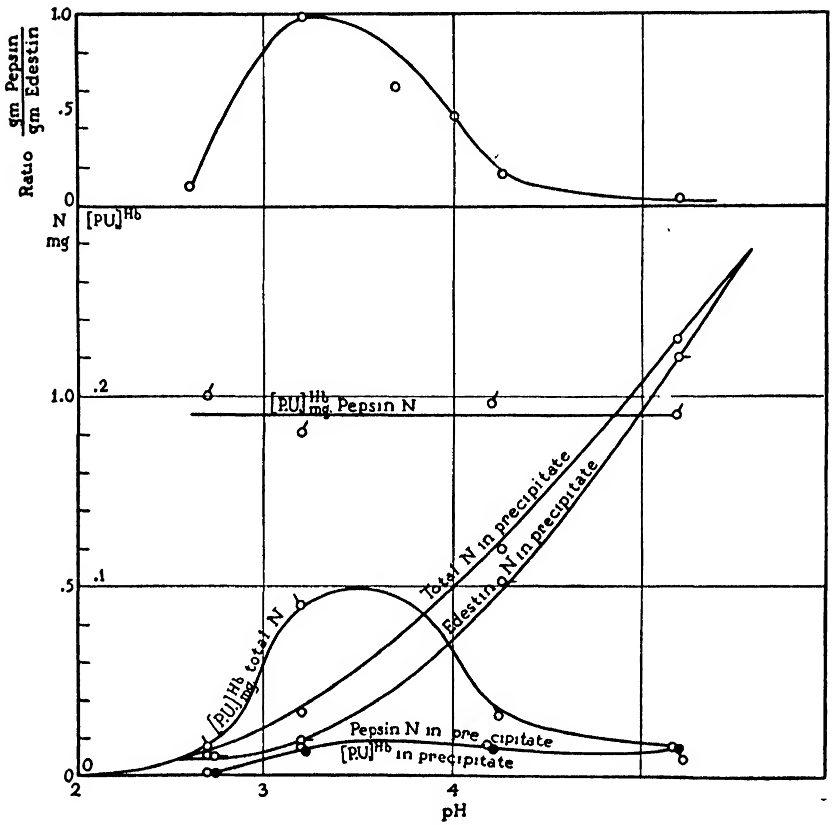

FIG. 6. Formation of edestin-pepsin from edestin and pepsin solutions at various pH.

IX. Effect of the Relative Concentrations of Pepsin and Edestin on the Composition of Edestin-Pepsin at pH 3.8 and 0°C.

Experimental Procedure.—Solutions of pepsin and edestin containing 2 mg. nitrogen per ml. each, both at pH about 3.8 prepared and cooled to 0°C. A series of tubes was prepared containing 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 ml. pepsin solution and the total volume of the various tubes made up to 10 ml. with edestin solution. The tubes were kept at 0°C. for 1 hour, centrifuged and the precipitates washed with 10 ml. water and dissolved in 10 ml. N/20 hydrochloric acid and analyzed for total nitrogen, pepsin nitrogen and peptic activity by the hemoglobin method. The edestin nitrogen was calculated as the difference between total protein nitrogen and pepsin nitrogen. The results were calculated on the basis of 1 ml. of the original suspension.

The results of an experiment in which the relative quantity of pepsin and edestin were varied is shown in Fig. 7. Here the composition of the precipitate has been plotted against the composition of the original solutions. As the concentration of pepsin increases, the quantity of precipitate, its specific activity and its total activity, as well as the quantity of pepsin nitrogen, all increase and reach a maximum corresponding to equivalent amounts by weight of edestin and pepsin. As the concentration of pepsin is increased still further the quantity of the precipitate decreases but its specific activity and the proportion of pepsin in it increase slightly and then stay constant. The precipitate formed under these conditions consists of nearly three equivalents pepsin per equivalent edestin or three parts pepsin by weight per one of edestin since pepsin and edestin have the same equivalent weight. This corresponds to nearly 15 moles of pepsin per mole of edestin.

The results indicate that a definite compound is formed between the edestin and the pepsin. The isoelectric point of edestin is about pH 6.0 while that of pepsin is about 2.7 (11) so that within this range of acidity the edestin is present as a positive ion while the pepsin is present as a negative ion. According to Hitchcock (12) 0.45 gm. of edestin combines with 0.55 milliequivalents of acid. 1 gm. of edestin is slightly more, therefore, than 1 milliequivalent. 1 gm. of pepsin combines with 1.1 milliequivalent of alkali so that 1 gm. of pepsin is also slightly more than 1 milliequivalent. It is possible to determine from the titration curves of the two proteins what per cent of the protein is ionized at any pH. If this is done and it be further assumed that the positive edestin ions react with the negative pepsin ions to form a slightly soluble compound, then the position of the maximum near pH 4.0 is correctly predicted. Since the precipitate, however, has varying composition it is not possible to account for its formation on this simple basis. If a series of compounds is assumed it is possible to fit the curves quite closely but so many arbitrary constants are involved that the results are not very convincing. It is evident, however, as the writer has pointed out previously (4), that the formation of this complex is not very closely connected with the hydrolysis of the edestin since the pH corresponding to the maximum complex formation is around 4.0 while the maximum from the rate of digestion is near pH 2.0. It is probable that the reaction is quite similar to that between proteins and ordinary nucleic acids (14) and is not especially connected with the proteolytic activity of the pepsin. Unfortunately experiments cannot be carried out with inactivated pepsin since inactivated pepsin is insoluble through this range of pH.

It may be pointed out that in the preceding experiments the concentrated solutions of commercial pepsin used were highly supersaturated with regard to

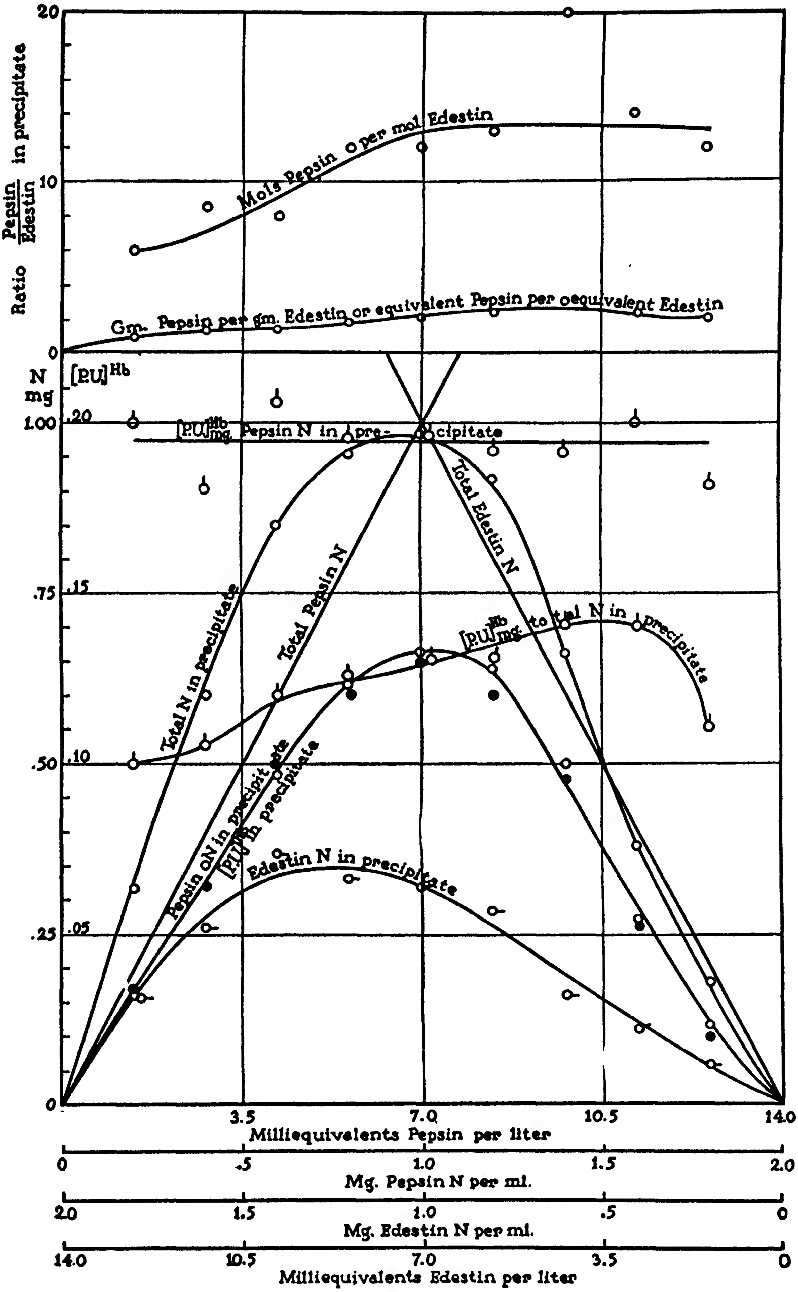

FIG. 7. Effect of the relative quantities of pepsin and edestin on the formation of edestin-pepsin at pH 4.0.

pepsin since the solubility of crystalline pepsin is quite low from pH 2.5 to pH 4.0. If concentrated solutions of crystalline pepsin had been used the results would have been entirely different since the pepsin would be precipitated from the more acid solutions even without the edestin. Commercial preparations of pepsin contain some substances, probably protein split products, which prevent the precipitation of the pepsin protein and render very highly supersaturated solutions quite stable. However, if a 10 per cent solution of Cudahy pepsin, between pH 3.0 and 4.0 is inoculated with pepsin crystals and allowed to stand at 6°C. for several weeks quite an appreciable quantity of pepsin crystallizes out.

X. *Absorption of Pepsin by Melon (Cucumis) Globulin (15)*

The preceding experiments were done with edestin but exactly similar results may be obtained with globulin from melon seed (*Cucumis*) as reported by Waldschmidt-Leitz and Kofrányi (6).

TABLE VII

Absorption of Pepsin by Melon (Cucumis) Globulin

	Total nitrogen per ml.	Pepsin nitrogen per ml.	$[\mathrm{P.U.}]^{\mathrm{Hb}}_{\mathrm{ml.}}$	$[\mathrm{P.U.}]^{\mathrm{Hb}}_{\mathrm{mg.\ pepsin\ N}}$
	mg.	*mg.*		
Pepsin solution	0.067	0.056	0.009	0.16
First supernatant	0.054	0.050	0.007	0.14
Second supernatant	0.037	0.042	0.007	0.17
Third supernatant	0.066	0.024	0.005	0.20
Combined precipitates		0.030	0.005	0.165

Experimental Procedure.—100 ml. of a solution of twice crystallized pepsin containing 0.37 mg. pepsin per ml. pH about 3.5 cooled to 0°C. and 100 mg. of crystalline *Cucumis* globulin added. The suspension was stirred for 10 minutes and centrifuged. 100 mg. of globulin was added to the supernatant and the process repeated. The supernatant was again extracted with 100 mg. of globulin. The precipitates were combined, dissolved with N/50 hydrochloric acid, the solution titrated to about pH 2.0 with hydrochloric acid and made up to 100 ml. and allowed to digest at 37°C. for 4 hours. The supernatant solutions and the solution of the precipitate were then analyzed for total nitrogen, pepsin nitrogen and peptic activity by the hemoglobin method.

The results of an experiment in which a solution of crystalline pepsin was treated with successive quantities of melon globulin are shown in Table VII. As the table shows, about half the total activity

is removed from the solution by the crystalline globulin and at the same time a corresponding quantity of pepsin nitrogen is also removed. The total nitrogen content of the solution decreases at first and then increases as some of the globulin dissolves and in this particular experiment it happens to be practically the same after three extractions as it was originally. This is the result which was reported by Waldschmidt-Leitz and Kofrányi from dry weight determinations and which led them to the conclusion that the active group had been removed from the pepsin protein and the inert protein left in solution. As the present experiments show, however, this is not the case. A quantity of pepsin protein equivalent to the activity removed is taken up by the foreign protein and there is no evidence that the pepsin is decomposed into an inert protein and an active group.

TABLE VIII

Absorption of Pepsin by Gelatin at Various pH

pH.	4.65	4.65	4 0	3.4	3 0	2 0
Gelatin, *gm*	0	2.5	1.0	1.0	1.0	1.0
Pepsin solution, *ml*	0.5	0.5	1.0	1.0	1.0	1.0
$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$ supernatant	0.0074	0.0029	0.0071	0.0091	0.0083	0.013
$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$ gelatin		0.05	0.034	0.024	0 022	0.016
Pepsin nitrogen in gelatin, *mg*	0	0.25	0.16	0.13	0.10	0.078
$[\text{P. U.}]^{\text{Hb}}_{\text{mg.}}$ pepsin nitrogen in gelatin		0.20	0.21	0.185	0.22	0.20
Ratio $\dfrac{[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}\ \text{gelatin}}{[\text{P. U.}]^{\text{Hb}}_{\text{ml}}\ \text{supernatant}}$		17.0	5.0	2.6	2.6	1.2

XI. Absorption of Pepsin by Gelatin at Various pH

Experimental Procedure.—A series of suspensions of powdered isoelectric gelatin in various concentrations of hydrochloric acid, total volume 50 ml. was prepared and cooled to 6°C. for ½ hour. 1 ml. of dilute crystalline pepsin solution was added and the suspension stirred for ½ hour and filtered. The gelatin precipitates were melted at 37°C. and the solutions analyzed for pepsin nitrogen by precipitation with hot trichloracetic acid and peptic activity by the hemoglobin method.

In the experiments with gelatin reported previously (4), the activity alone was followed and no determinations were made of the changes in pepsin nitrogen since at that time it was not known that the activity

was a property of the pepsin protein. The experiments have, therefore, been repeated and determinations made of the pepsin protein in the gelatin as well as of the activity. The results of the experiment are shown in Table VIII. As in the experiments with edestin, the quantity of pepsin protein taken up by the gelatin is just equivalent to the activity found in the gelatin so that in this case also the pepsin protein itself is taken up by the foreign protein. It will be noted that much more is taken up with isoelectric gelatin than by acid gelatin. This peculiarity was noted before (4) and was found to be due to surface adsorption on isoelectric gelatin whereas with acid gelatin the quantity taken up was independent of the surface.

XII. Inactivation of Pepsin by Alkali in the Presence of Edestin

Warburg and Christian (13) have found that the respiratory ferment decomposes into a protein and a non-protein fraction when the protein is denatured. Hemoglobin also possesses the same peculiarity. It seemed possible, therefore, if the pepsin were denatured by alkali in the presence of edestin, which is not affected by dilute alkali, that the active group might leave the pepsin and become attached to the edestin. In this case it would be expected that a loss in activity of pepsin solutions containing edestin in alkali would be less than the loss in activity of pure pepsin solutions at the same pH. This, however, is not the case as shown in Table IX. Evidently either pepsin does not decompose into two parts when the pepsin protein is denatured or if it does the prosthetic group has no activity under these conditions and edestin cannot replace the pepsin protein. The fact that active pepsin can be prepared from pepsin denatured by alkali indicates that splitting of the molecule does not occur although the yield of active pepsin is so small as to render this argument more or less inconclusive.

TABLE IX

Inactivation of Pepsin by Alkali in the Presence of Edestin

pH.		5 0	5 6	8.0
$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$	"Edestin pepsin"	0 0074	0.0072	0.001
	Pepsin solution	0.0075	0.0073	0.0008

Experimental Procedure.—100 mg. of edestin suspended in 10 ml. of water and 1 ml. dilute crystalline pepsin solution added. Increasing amounts of alkali added to a series of these tubes and the suspension allowed to stand at 25°C. for 10 minutes. Control series without edestin prepared in the same way. The pepsin activity determined by the hemoglobin method.

XIII. Spheroidal Pepsin

Dyckerhoff and Tewes found that an active precipitate could be obtained from autolyzed edestin-pepsin or from partly autolyzed pepsin solutions which appeared in the form of spherical, highly refractile granules which they called "*kugeln*" pepsin. These spheroids are

TABLE X

Spheroids

Fraction No		(1) Spheroids	(5) Crystals plus amorphous	(6) Filtrate	Original twice crystallized pepsin
N/ml., *mg.*	Total	12.0	0.60	4.1	
	Protein	9.5	0.45	3.7	
$[P. U.]^{Hb}$	/ml.	1.8	0.066	0.71	
	/mg. N	0.15	0.11	0.17	0.17
	/mg. protein N	0.19	0.15	0.19	0.19
$[P. U.]^{Gel\ V^-}$	/ml.	160	20	56	
	/mg. N	13.3	33	13.5	10
	/mg. protein N	17	44	15	12
$[P. U.]^{Cas\ V^-}$	/ml.	11,500	380	3,800	
	/mg. N	960	630	930	1,000
	/mg. protein N	1,200	850	1,030	1,100

characteristic of proteins which are not sufficiently purified or which appear under unfavorable conditions for crystallization. The spheroids obtained from autolyzed "edestin-pepsin" solutions consist, as described under Experiment 2, almost entirely of pure pepsin with varying amounts of some form of non-protein nitrogen. The same precipitate may be obtained from pepsin solutions which have been allowed to stand and which are too acid and too dilute for crystallization to take place readily. They may be obtained as characteristic pepsin crystals by dissolving with alkali, precipitating rapidly in the amorphous form with sulfuric acid and crystallizing as usual. The results of such an experiment are shown in Table X.

Experimental Procedure.—15 gm. twice crystallized pepsin filter cake dissolved in 200 ml. water at 45°C. and 5 ml. N/2 sodium hydroxide added (clear solution); 10 ml. N/2 sulfuric acid added, precipitate forms (pH 2.4) and suspension left at 6°C. for 24 hours. Precipitate small spheroids, filter, 10 gm. cake (1). 50 ml. water and 4 ml. N/2 sodium hydroxide added to (1) (clear solution). 3 ml. N/2 sulfuric acid added, slight precipitate, (spheroids), suspension cooled slowly to 20°C. Filter, precipitate (2) 5 gm. (2) spheroids dissolved in 15 ml. water and 1 ml. N/2 sodium hydroxide added (clear solution) (3). (3) titrated to pH 3.0 with sulfuric acid and cooled to 6°C., amorphous precipitate, filter and precipitate dissolved at 45°C. with sodium hydroxide. Solution titrated to pH 3.5 with sulfuric acid, cooled slowly to 6°C. for 4 days. Precipitate of poor crystals formed. Filter and precipitate dissolved in N/50 pH 5.0 acetate (5). Filtrate (6).

The activity was determined by several methods. The specific activity calculated on the basis of protein nitrogen is constant for the hemoglobin and casein method in all the fractions but is slightly low as calculated on the basis of total nitrogen. The specific activity, as determined by the gelatin viscosity, is high as is always the case when a small fraction is precipitated from incompletely purified pepsin solutions since the gelatinase is concentrated in such precipitates.

The analytical work reported in this paper was done by Mr. N. Wuest.

SUMMARY

Crystalline proteins, such as edestin or melon globulin, remove pepsin from solution. The pepsin protein is taken up as such and the quantity of protein taken up by the foreign protein is just equivalent to the peptic activity found in the complex. The formation of the complex depends on the pH and is at a maximum at pH 4.0.

An insoluble complex is formed and precipitates when pepsin and edestin solutions are mixed and the maximum precipitation is also at pH 4.0. The composition of the precipitate varies with the relative quantity of pepsin and edestin. It contains a maximum quantity of pepsin when the ratio of pepsin to edestin is about 2 to 1. This complex may consist of 75 per cent pepsin and have three-quarters of the activity of crystalline pepsin itself. The pepsin may be extracted from the complex by washing with cold N/4 sulfuric acid. If the complex is dissolved in acid solution at about pH 2.0 the foreign protein is rapidly digested and the pepsin protein is left and may be isolated.

The pepsin protein may be identified by its tyrosine plus tryptophane content, basic nitrogen content, crystalline form and specific activity.

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CRYSTALLINE PEPSIN

VI. INACTIVATION BY BETA AND GAMMA RAYS FROM RADIUM AND BY ULTRA-VIOLET LIGHT

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Previous work (1) has shown that when solutions of crystalline pepsin are inactivated by alkali or by heat the loss in activity is exactly proportional to the loss of native protein. These experiments confirm the idea, therefore, that the native protein molecule is the active enzyme. Proteins are denatured (2) by exposure to radium or to ultra-violet light and it is also known that pepsin solutions (3) are inactivated under these conditions. Inactivation of the enzyme by radium or ultra-violet light, therefore, furnishes another method of testing the relationship between the protein and the active molecule. If the protein molecule itself is responsible for the activity then any loss in activity must be accompanied by a corresponding decrease in the protein. On the other hand, if a hypothetical, active molecule is merely associated with the native protein there is no reason to suppose that the rate of inactivation of the active molecule would be the same as the rate of denaturation of the protein. The inactivation of pepsin solutions has been studied from this point of view and it has been found that the loss in activity is just proportional to the loss in native protein when the enzyme is inactivated either by radium or by ultra-violet light. These results, therefore, furnish additional evidence in favor of the idea that the protein molecule itself is responsible for the activity.

EXPERIMENTAL RESULTS

I. Decrease in Activity of Protein Nitrogen of Pepsin Solutions Exposed to Radium Bromide at pH 5.0 and 0°C.

The results of an experiment in which pepsin solutions of various concentrations were exposed to the radiation of 100 mg. of radium

bromide at 0°C. are shown in Table I. The decrease in activity, as determined by the hemoglobin method, is just proportional to the

TABLE I

Changes in Activity and Protein Nitrogen of Pepsin Solutions Exposed to Radium Bromide, pH 5.0, 0°C.

hrs.									
	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$	0.5	0.2	0.043	0 048	0 010	0 010	0.0048	0 0048
0	P N/ml., *mg.*	2.6	1.0	0.24	0 22	0.050	0.050	0.023	0.023
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$	0.19	0.20	0.21	0.22	0.20	0.20	0.21	0.21
	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$							0.0041	
25	P N/ml., *mg.*							0.020	
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$							0.20	
	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$			0.037					0.0036
72	P N/ml., *mg.*								0.019
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$								0.19
	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$				0.031		0 0054		
96	P N/ml., *mg.*				0.16		0.030		
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$				0.19		0.18		
	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$	0.5		0.021		0.0056			
160	P N/ml., *mg.*	2.5		0.10		0 035			
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$	0.20		0.21		0.15			
	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$		0.17						
190	P N/ml., *mg.*		0.78						
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$		0.22						
	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$	0.43							
310	P N/ml., *mg.*	2.17							
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$	0.20							
	$[\text{P. U.}]^{\text{Hb}}_{\text{ml.}}$		0.083						
770	P N/ml., *mg.*		0.48						
	$[\text{P. U.}]^{\text{Hb}}_{\text{mg.PN}}$		0.17						

decrease in the protein nitrogen of the solution as shown by the fact that the activity per mg. protein nitrogen remains constant. No denatured protein appears in the solution although it is probable

that the first step in the reaction is the formation of denatured protein. The rate of denaturation under these conditions, however, is extremely slow and the denatured protein, if present, would undoubtedly be digested by the remaining active native protein as rapidly as it was formed and so does not accumulate in the solution.

Effect of the Concentration of Pepsin

Below about 0.05 mg. nitrogen per ml. the per cent inactivated is nearly constant, while in more concentrated solutions the actual quantity inactivated is approximately constant. Similar results were obtained by Hussey and Thompson (3). They indicate that, under the conditions of the experiment most of the energy is absorbed by 0.05 mg. nitrogen per ml. so that increasing the concentration beyond this point does not have much effect upon the number of protein molecules inactivated.

Experimental Procedure

A solution of three times crystallized pepsin was prepared in pH 5.0 N/20 acetate buffer and diluted to the concentrations noted in the table with N/20 acetate buffer. 25 ml. of this solution was placed in a 1.5 cm. centrifuge tube and a glass tube (with 0.5 mm. walls) containing 100 mg. of radium bromide suspended in the center of the solution. The tube was kept in the ice box at 0°C. and 1 ml. samples removed and analyzed for protein nitrogen and activity as noted in the table.

Activity Determinations.—The activity determinations were made with hemoglobin by the method of Anson and Mirsky(4).

Protein Nitrogen Determination.—1 ml. of solution added to 5 ml. of 5 per cent boiling trichloracetic acid, the precipitate centrifuged and washed three times with 5 per cent trichloracetic acid, and total nitrogen in the precipitate determined.

Test for Denatured Protein.—1 ml. of solution added to 10 ml. of N/2 sodium sulfate and N/20 sulfuric acid. Any denatured protein precipitates under these conditions and some of the samples which had been almost completely inactivated gave a slight cloud. The amount of denatured protein was, however, too small to determine.

II. Inactivation by Ultra-Violet Light

A. Changes in Protein Nitrogen and Activity of Pepsin Solutions at Various pH Exposed to Ultra-Violet Light

The results of an experiment in which pepsin solutions at various pH were exposed to light from a mercury arc are shown in Table II. As in the case of radium inactivation practically no denatured protein

TABLE II

Change in Activity and Protein Nitrogen in Pepsin Solutions of Various pH Exposed to Ultra-Violet Light

pH........	0			1.7			3.0			4.65		
Buffer.....	1.0 N hydrochloric acid			N/50 hydrochloric acid			N/65 acetic acid			N/50 4.65 acetate		
Time	$[P.U.]^{Hb}_{ml.}$	P N/ml.	$[P.U.]_{mg. PN}$	$[P.U.]^{Hb}_{ml.}$	P N/ml.	$[P.U.]_{mg. PN}$	$[P.U.]^{Hb}_{ml.}$	P N/ml.	$[P.U.]_{mg. PN}$	$[P.U.]^{Hb}_{ml.}$	P N/ml.	$[P.U.]_{mg. PN}$
hrs.		*mg.*			*mg*			*mg.*			*mg.*	
0	0.019	0.098	0.20	0.020	0.098	0.21	0 020	0.098	0.22	0.020	0.098	0.21
2.55	0.014	0.076	0.18	0.015	0.070	0.215	0.017	0.080	0.21	0.017	0.084	0.20
5.00	0 0092	0 058	0.16	0.0095	0.050	0.19	0 0118	0 070	0.17	0.013	0.078	0.17
8.75	0.0054	0.030	0.18	0.0050	0 018	0.28	0 0077	0 058	0 13	0.0086	0.063	0.14

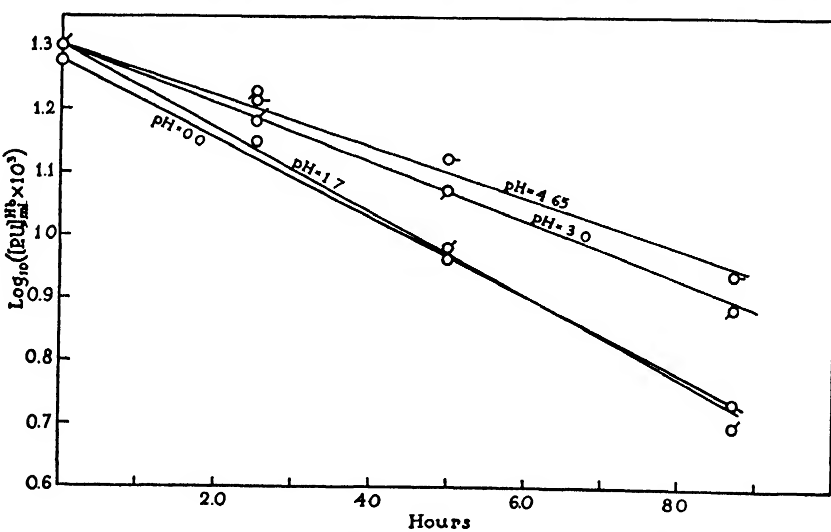

FIG. 1. Inactivation of pepsin by ultra-violet light at different pH

appears in the solution and the loss in activity is accompanied by a corresponding decrease in the total protein nitrogen of the solution; *i.e.*, the activity per mg. protein nitrogen remains constant throughout the experiment. The rate of inactivation depends upon the pH and is a maximum at about pH 2.0 and decreases as the pH becomes more

alkaline. The pH corresponding to the maximum rate of inactivation agrees with that found by Collier and Wasteneys (5) and is slightly less acid than that reported by Pincussen and Vehara (3). The reaction follows approximately a monomolecular course, as shown in Fig. 1, in which the log of the activity is plotted against the time in hours.

Experimental Procedure

A solution of three times crystallized pepsin was prepared in N/20 pH 4.65 acetate. It contained 2.5 mg. protein nitrogen per ml. 1 ml. of this solution was diluted with 25 ml. of the buffer noted in the table and the pH determined. 25 ml. of the solution was placed in 1 cm. quartz test-tubes arranged in a semicircle around a General Electric "Lab-Arc" at a distance of 8.5 cm. from the arc. The lamp was operated on 110 volts A.C. and 1.8 amperes and was allowed to run 1 hour before the experiment was started. The activity and protein nitrogen were determined, as described for the radium experiments, except that with very dilute pepsin solutions it was necessary to use 5 ml. for the protein nitrogen determination. Control tubes containing 25 ml. of the solution in glass test-tubes were placed beside the quartz tubes. There was no change in activity or protein nitrogen in the solution in the glass tubes showing that the inactivation was due entirely to ultra-violet light. The temperature of the solutions was 15°C. The positions of the quartz tubes were interchanged at intervals of about 20 minutes so that any local variations in the light intensity were distributed. Special control experiments showed that the rate of inactivation was the same in the various tubes.

The analytical work was done by Mr. Nicholas Wuest.

SUMMARY

1. The loss in activity of crystalline pepsin solutions when exposed to beta and gamma rays from radium or to ultra-violet light is accompanied by a corresponding decrease in pepsin protein.

2. The rate of inactivation by ultra-violet light depends upon the pH and is a maximum at about pH 2.0.

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MOLECULAR WEIGHT, MOLECULAR VOLUME, AND HYDRATION OF PROTEINS IN SOLUTION

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The gram molecular weight and volume of a dissolved substance may be calculated from the osmotic pressure of the solution. Osmotic pressure is affected only slightly by hydration and so furnishes no precise information as to the size of the hydrated molecule as it exists in a solution. The radius of the hydrated molecule in solution, and hence the gram molecular volume of the hydrated solute, may be determined from diffusion measurements. The difference between this figure and the gram molecular volume, as found by osmotic pressure, therefore represents the amount of hydration. The hydration may also be calculated from viscosity measurements. These two independent methods for the estimation of hydration give essentially the same values for the hydration of crystalline hemoglobin and crystalline trypsin.

Molecular Weight from Osmotic Pressure

The gram molecular weight of a substance in solution may be defined as that quantity of dry substance which, when dissolved in 1 liter of solvent, gives rise to an osmotic pressure of 22.4 atmospheres at 0°C. If the osmotic pressure of a solution is known, therefore, its molar concentration may be calculated. Since there are 6.06×10^{23} molecules in a gram molecule the average weight of the individual molecules may be found if the weight concentration of the solution is also known. This figure represents the average dry weight of the individual molecules of solute for which the membrane is impermeable but furnishes no definite information as to their size. Solvation of the molecules increases their size but does not change the number of molecules and affects the osmotic pressure only by decreasing the quantity of free solvent. This decrease in the quantity of free solvent

is not noticeable experimentally except in concentrated solutions or when the solvation is large.

Calculations of the molecular weight from osmotic pressure determinations involve the following assumptions:[1]

1. The system is at equilibrium.
2. The membrane is permeable to the solvent but impermeable to the solute in question.
3. The osmotic pressure is proportional to the concentration (van't Hoff's law).
4. The molecules of solute are all of the same size.

In the case of collodion membranes and aqueous solutions of proteins the first three conditions are fulfilled but the fourth may or may not be true. The protein molecules themselves may vary in size and in addition they may be combined with small ions or molecules which are thus prevented from free diffusion through the membrane, as in the Donnan equilibrium. In this case the osmotic pressure is due to both the protein molecules and the excess concentration of inorganic ions and the value calculated for the molecular weight represents the average of these various molecular species present. The complication due to Donnan equilibrium may be avoided experimentally by measurements made at the isoelectric point of the protein. The effect of neutral salts also furnishes a test for the presence of such Donnan pressures.

Radius of Molecules from Diffusion Measurements

The radius of the molecule determines the rate of diffusion in accordance with Einstein's equation (1)

$$D = \frac{R\,T}{N}\,\frac{1}{6\pi r\eta}$$

R = gas constant
T = absolute temperature
N = Avogadro's number 6.06×10^{23}
η = viscosity of solvent
r = radius of molecule
molecular volume = $4/3\ \pi\, r^3 N$

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[1] For a discussion of the osmotic pressure of hemoglobin solutions see Adair, G. S., *Proc. Roy. Soc. London, Series A*, 1928, **120**, 573.

Thus, if the diffusion coefficient of the solute is known the radius of the molecules and hence the gram molecular volume may be calculated. This value for the radius represents the radius of the particle which actually moves in the solution and therefore includes any solvent carried with the molecule. The following assumptions are involved in Einstein's equation:

1. The diffusing particles are few and large compared to the molecules of the solvent.
2. They are spherical.
3. They are electrically neutral.
4. They are impelled by a force equal to the osmotic pressure as given by van't Hoff's law against a resistance as given by Stokes' law.

As in the case of osmotic pressure the effect of ionization is the most important source of error with protein solutions. The presence of charged molecules may again be tested for by determining the effect of neutral salts and of the pH. If the molecules are not of the same size a constant value for the diffusion coefficient will not be obtained but the value will decrease as the experiment proceeds since the smaller particles will diffuse out faster. It is important, therefore, to continue the experiment until a large proportion of the solvent has diffused out; or better, to repeat the measurement on the first part of the diffusate, in order to be sure that the diffusion coefficient is actually the same for all of the solute. Otherwise entirely erroneous values may be obtained. The determination may be made conveniently and accurately as described by Northrop and Anson (2).

Calculation of Hydration from Osmotic Pressure and Diffusion Measurements

If the osmotic pressure and the diffusion coefficient of a solution are known, then the degree of hydration of the molecules of the solute can be determined as follows:

Let M be the gram molecular weight of the dissolved substance as determined from osmotic pressure measurements,
r the average radius of the molecules as determined from diffusion measurements,
S the specific volume of the dry substance,

then the gram molecular volume of hydrated molecules equals $4/3\,\pi r^3 N$ and the gram molecular volume of non-hydrated molecules equals $S\,M$.

Volume of water of hydration (if water is used as solvent) equals

$$4/3\ \pi r^3 N - S\ M$$

and

$$\frac{4/3\ \pi r^3 N - S\ M}{M}$$

equals volume of water of hydration per gram of dry solute, or

$$\frac{4/3\ \pi r^3 N - S\ M}{N}$$

equals volume of water of hydration per molecule solute.

Determination of Hydration from Viscosity Measurements

An independent method for the determination of the amount of hydration of substance in solution is the measurement of viscosity (3). This method applies to the case of molecules or particles large as compared with the size of the molecules of the solvent and consists in determining the relative viscosity of the solution as compared with the viscosity of the solvent. The volume of the solute may be calculated by aid of the empirical formula

$$\eta = \frac{1 + 0.5\ \phi}{(1 - \phi)^4}$$

where η equals the relative viscosity of solution and ϕ equals the volume of solute expressed as the fraction of the total volume of the solution. The formula was found to hold well for a large number of solutions or dispersions of molecules of relatively large size.

The two methods of determining the degree of hydration were used here in the case of such substances as hemoglobin and crystalline trypsin, and the results show that there is quite a close agreement between the two methods.

The results are summarized in the following table:

	Molecular weight	Average radius of hydrated molecule	Water of hydration per gm. dry wt.	
			Osmotic pressure diffusion method	Viscosity method
			$cm.^3$	$cm.^3$
Hemoglobin...............	67,000	$2.73^* \times 10^{-7}$	0 to 0.14	0.13†
Isoelectric gelatin..........	61,500	(5.4×10^{-7})	(5.8)	5.9
Crystalline trypsin.........	35,000	2.6×10^{-7}	0.54	0.49

Technique.—Osmotic pressure, Northrop and Kunitz (4), Adair (5); diffusion, Northrop and Anson (2).

* Svedberg, T. (Colloid chemistry, American Chemical Society Monographs, New York, The Chemical Catalog Co., 2nd edition, 1928, 165) obtains a value of 0.0342 cm.^{2}/day corresponding to a molecular radius of 3.35×10^{-7} cm. Svedberg's measurements were made while the molecules were moving under the influence of centrifugal force and the difference in the value may be due to the fact that the molecules are not spherical. In this case they might be oriented in a gravitational field and would, therefore, move through the liquid at a rate different from that determined by diffusion alone.

† Adair and Robinson (*J. Physiol.*, 1931, **72**, 28) obtained a value of 0.2 ml. water per gm. hemoglobin from measurements of the water absorbed by the dry protein from ammonia sulfate solutions.

EXPERIMENTAL

Hemoglobin

Osmotic Pressure.—Measurements of Adair (5).

Diffusion.—Northrop and Anson (2).

Viscosity.—Measurements were made of the viscosity at 5°C. of various concentrations of CO-hemoglobin in M/20 phosphate buffer pH 6.8 using an Ostwald viscosimeter; specific volume of dry hemoglobin equals 0.75.

The data given in Table I show that the hydration of hemoglobin under the conditions of the experiment decreases with the dilution and is about 0.1 ml. per gm. of hemoglobin at concentrations below 2 per cent. The diffusion experiments of Northrop and Anson were done under the same conditions of hemoglobin in the range of 1–2.5 per cent. The experiments of Northrop and Anson show that at 5°C. the diffusion coefficient for 2.5 per cent hemoglobin in N/20 phosphate buffer pH 6.8 is between 0.0434 and 0.0401 cm.^{2}/day. The calculated radius of the molecules is between 2.65×10^{-7} and 2.86×10^{-7}. Hence $4/3\pi r^3 N$ equals between 47,300 cm.^{3} and 59,500 cm.^{3} From osmotic pressure measurements (Adair) $S\ M = 67{,}000 \times 0.75 = 50{,}000$.

Volume of water of hydration per mole = between 0 and 9,500 cm.^{3}
" " " " " " gram = " 0 " 0.14 cm.^{3}

Thus it is seen that in the case of hemoglobin the amount of water of hydration per gram of protein, as obtained by viscosity measurements,

is so small as to be within the experimental error of the diffusion measurements.

Crystalline Trypsin

Osmotic Pressure Measurements.—Northrop and Kunitz (6).

Diffusion Measurements.—Scherp (7).

Viscosity.—Viscosity measurements of solutions of crystalline trypsin were made under conditions similar to those employed in the determination of the molecular weight of crystalline trypsin by means

TABLE I

Viscosity Measurements of CO-Hemoglobin, pH 6.8 at 5°C.

Concentration of protein	Relative density at 5°C.	Relative viscosity*	Calculated volume of solute in cm.³/100 cm.³ solution	Specific volume per gm. protein	Volume of water of hydration per gm. hemoglobin
gm /100 ml. solution				*cm* ³	*cm* ³
2.10	1.006	1.084	1.85	0.88	0.13
4.20	1.012	1.175	3.65	0.87	0.12
6.30	1.018	1.290	5.60	0.89	0.14
8.36	1.024	1.445	7.90	0.95	0.20
10.45	1.030	1.610	10.15	0.97	0.22

* These values are much lower than those reported by Lewis and Loughlin (*Biochem. J.*, London, 1932, **26**, 480) and give rise to correspondingly lower values for the hydration. This difference is not due to the salt present since repetition of the measurements with salt-free hemoglobin solution gave practically the same figures for the viscosity of the solution as found for hemoglobin solution in M/2 phosphate buffer.

of osmotic pressure measurements, as well as in the determination of the diffusion coefficient of crystalline trypsin as carried out by Dr. Scherp in this laboratory.

The procedure was as follows. A solution of crystalline trypsin in M/10 acetate buffer pH 4.0 was made salt-free by dialysis in the cold room against N/10,000 hydrochloric acid. The dialyzed trypsin was then diluted with equal volume of saturated magnesium sulfate in M/10 acetate buffer pH 4.0 and dialyzed against a definite volume of trypsin-free 0.5 saturated magnesium sulfate in M/10 acetate buffer pH 4.0 until equilibrium was established as indicated by the reading of a manometer tube inserted in the collodion bag containing the

trypsin solution. The outside solution was found to be free of any trypsin. A series of dilutions was then made of the trypsin solution by means of the outside magnesium sulfate solution and viscosity measurements were made at 5°C. The results are shown in Table II. The specific volume of dry trypsin was taken as 0.75 ml./gm. which was found to be common for proteins of the albumin type. The average value of the water of hydration of crystalline trypsin when dissolved in 0.5 saturated magnesium sulfate pH 4.0 was thus found by the viscosity measurements to be 0.5 ml. per gm. dry protein.

TABLE II

Viscosity at 5°C. of Various Concentrations of Crystalline Trypsin in 0.5 Saturated Magnesium Sulfate and M/10 Acetate Buffer pH 4.0

Concentration of trypsin	Time of outflow	Relative viscosity	Calculated volume of hydrated trypsin in cm.³/100 cm.³ solution	Volume of hydrated trypsin per gm. dry trypsin	Water of hydration per gm. dry trypsin
gm /100 ml.	*sec.*			*cm.³*	*cm.³*
0	203.4	1.000	0		
0.8	212.6	1.045	1.00	1.25	0.50
1.6	221.5	1.089	2.00	1.25	0.50
2.4	231.0	1.135	2.90	1.21	0.44
3.2	241.4	1.187	3.90	1.22	0.47
4.0	257.0	1.265	5.15	1.29	0.54
Average					0.49

The radius of hydrated trypsin molecules under the same conditions, as determined by Scherp from diffusion measurements, was found to be 2.6×10^{-7} cm. The volume of one mole of hydrated trypsin is therefore

$$(2.6 \times 10^{-7})^3 \times 4/3\,\pi \times 6.06 \times 10^{23} = 44{,}700 \text{ cm.}^3$$

The molecular weight of the trypsin in solution under the same conditions was found by osmotic pressure measurements to be about 35,000 gm. The molecular volume of the non-hydrated trypsin equals 26,000 cm.³ Hence, water of hydration per mole of trypsin equals 19,000 cm.³ Water of hydration per gram dry trypsin equals 19,000/35,000 equals 0.54 cm.³/gm. Thus, the value for hydration

of trypsin, as determined by diffusion experiments in connection with osmotic pressure measurements checks quite closely with the value obtained by viscosity measurements. This agreement serves as a check for the viscosity formula and justified the application of Einstein's diffusion formula to protein solutions.

Gelatin

The hydration of gelatin, as calculated from osmotic pressure and from viscosity measurements, has been described in a previous paper (8). The value of the hydration so obtained was 6 $cm.^3$ water per gram dry gelatin in 3 to 5 per cent solutions.

Diffusion measurements were made with gelatin solutions in order to see whether the hydration, as determined by this method in connection with the osmotic pressure measurements, agrees with that calculated from viscosity. If a 5 per cent solution of gelatin pH 4.7 in M/1000 acetate buffer was allowed to diffuse, a constant value for the diffusion coefficient of 0.05 $cm.^2$/day was obtained. However, if the first diffusate was replaced in the cell and the experiment repeated, a much larger value for the diffusion coefficient was found. Gelatin solutions, therefore, as was to be expected, are not homogeneous but the relative size of the particles or their relative amount, do not differ sufficiently to cause a noticeable drift in the diffusion coefficient as determined from any one experiment. Trial calculations show that a mixture containing 30 per cent of particles of radius 2 and 70 per cent of particles of radius 1 will diffuse in such a way as to give a value for the diffusion coefficient, as calculated from the total amount diffused, which does not vary over 10 per cent until more than 75 per cent of the total original quantity has diffused out. Such a mixture, however, would give an entirely different value for the diffusion coefficient if the measurements were repeated on the diffusate. This is the result obtained with the gelatin. The results are complicated in addition by the fact that some hydrolysis of the gelatin occurs during the experiment.

Since the value of the diffusion coefficient has no physical significance unless the diffusing particles are of nearly uniform size, the results with gelatin are of doubtful significance.

SUMMARY

1. The gram molecular weight of a substance may be calculated from the osmotic pressure of its solution.

2. The radius of the hydrated molecule and, hence, the gram molecular volume of the hydrated solute may be determined from diffusion measurements. The hydration of the molecules may, therefore, be calculated from osmotic pressure and diffusion measurements.

3. Hydration may also be determined by viscosity measurements. Hydration of crystalline hemoglobin, crystalline trypsin, and gelatin have been determined by these methods and found to be as follows:

	Molecular weight	Average radius of hydrated molecule	Water of hydration per gm. dry wt.	
			Osmotic pressure diffusion method	Viscosity method
			cm.[3]	*cm.*[3]
Hemoglobin................	67,000	2.73×10^{-7}	0 to 0.14	0.13
Isoelectric gelatin..........	61,500	(5.4×10^{-7})	(5.8)	5.9
Crystalline trypsin.........	35,000	2.6×10^{-7}	0.54	0.49

The results with gelatin calculated from the diffusion measurements are uncertain since gelatin solutions are not homogeneous.

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THE KINETICS OF PENETRATION

VI. Some Factors Affecting Penetration

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INTRODUCTION

Although sea water contains very much more sodium than potassium the latter predominates in the cells of many marine organisms. Presumably this is because it penetrates the protoplasm more rapidly.[1] A like situation in models affords a favorable opportunity to study certain variables which may be important in living cells.

In these models[2] electrolytes pass from an outer aqueous layer A (Fig. 1) through a non-aqueous layer B (representing the protoplasm) into an inner aqueous layer C (which may be called "artificial sap"); the latter consists at the start of distilled water and CO_2 bubbles in it throughout the experiment.

All three layers are stirred mechanically but at the phase boundaries are unstirred layers ($A_B B_o B_i$, and C_B) in which the movement of electrolytes depends on diffusion. The layers B_o and B_i, in which diffusion is slowest,[3] regulate the process of penetration.

In these experiments A contained KOH and NaOH which had been previously shaken up with a non-aqueous mixture consisting of 70 per cent guaiacol + 30 per cent *p*-cresol (which will be called G. C. mixture). As a result KOH combined with the constituents of the non-aqueous phase to form organic salts which may be lumped together[4] and called collectively KG, the corresponding designation for

[1] Osterhout, W. J. V., *Biol. Rev.*, 1931, **6**, 369; *Ergebn. Physiol.*, 1933, **35**, 967.

[2] Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 667.

[3] See p. 204. Also Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529.

[4] This introduces no serious error since the behavior of the organic salts seems to be very similar. *Cf.* footnote 2.

the sodium salts being NaG. We therefore have to do with the penetration of KG and NaG.

In the preceding paper[5] it was assumed that the rate of penetration of potassium is proportional to the concentration gradient in the non-aqueous layer B. This gradient may be regarded as $K'_o - K'_i$, where K_o represents the concentration of undissociated KG in the outer surface of B_o, and K'_i that in the inner surface of B_i (the corresponding designations for sodium are Na_o and Na'_i).

On reaching C, KG and NaG come in contact with CO_2 and form $KHCO_3$ and $NaHCO_3$. This raises the osmotic pressure in C so that

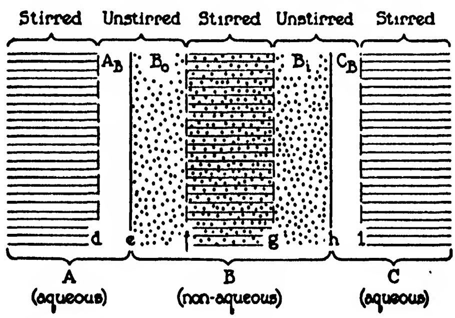

FIG. 1. Diagram of layers in the model. The aqueous phase A has an unstirred layer which is represented between d and e : from e to f is the corresponding unstirred layer in the non-aqueous phase B. Similar layers are present at the boundary between the non-aqueous phase B and the aqueous phase C.

water enters from A. Eventually a steady state is reached in which water and salts enter in a fixed ratio and the volume of C increases while its composition remains constant. This appears to be analogous to what happens in living cells.

Previous studies[6] indicate that the rate of entrance is proportional to $K'_o - K'_i$: it is also proportional to the diffusion constant. Hence we may write as an approximation

$$R_K = C_1 D_K (K_o - K_i)$$

[5] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529.

[6] This equation takes no account of the entrance of water. Regarding the significance of the "constants" see Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529.

Here R_K is the rate of entrance of moles of potassium into C and D_K the diffusion constant of undissociated KG in B. Using a corresponding notation for sodium we have

$$\frac{R_K}{R_{Na}} = \frac{C_1 D_K (K'_o - K'_i)}{C_2 D_{Na} (Na'_o - Na'_i)}$$

C_1 and C_2 are "constants"[6] which depend on the rate of stirring, the surface area, and shape of B, etc., and when they are the same for KG and NaG cancel out.

This equation permits only qualitative predictions because the ratio $(K'_o - K'_i) \div (Na'_o - Na'_i)$ changes during the process[7] and several disturbing factors intervene (p. 211).

Let us now consider K^o and K'_i. We may make the usual assumption that on each side of the phase boundary there are very thin layers in approximate equilibrium with each other. Hence if the total concentrations of potassium (KG + KOH + $KHCO_3$) in the aqueous surface layers be K_o and K_i we may put

$$\frac{K'_o}{K_o} = S_{K_o} \quad \text{and} \quad \frac{K'_i}{K_i} = S_{K_i},$$

where S_{K_o} and S_{K_i} are the partition coefficients or absorption coefficients.[8]

Hence we may put

$$\frac{R_K}{R_{Na}} = \left(\frac{D_K}{D_{Na}}\right) \frac{S_{K_o} K_o - S_{K_i} K_i}{S_{Na_o} Na_o - S_{Na_i} Na_i}$$

In view of this a knowledge of diffusion constants and partition coefficients becomes desirable.

Diffusion Constants

When only KG is present the meaning of the diffusion constant D_K requires no comment, but when we add NaG it will affect the value of

[7] The behavior of this ratio depends on the magnitudes of the partition coefficients as well as on their ratio. See p. 205.

[8] *Cf.* Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26**, 125; 1931–32 **29**, 993, 1234; *J Gen Physiol.*, 1928–29, **12**, 147, 407.

D_K. The divergence will be small in guaiacol (in which KG and NaG are very weak electrolytes)[9] but may be larger in water: this latter, however, does not concern us since for our purposes the diffusion in water may be regarded as negligible. Similar reasoning applies to the diffusion constant D_{Na}.

Let us now consider the ratio $D^K \div D_{Na}$. In order to determine this the diffusion apparatus of Northrop and Anson[10] was used. The upper part was filled with G. C. mixture containing equal concentrations of KG and NaG which were allowed to diffuse into the lower chamber containing G. C. mixture. The result showed that D_K is so close to D_{Na} that for our purposes they may be regarded as equal.

Similar tests in which water was employed as the solvent showed that KG and NaG diffuse ten to eleven times as fast in water as in G. C. mixture (this result is due in part to the higher viscosity of the G. C. mixture[11]). Hence the diffusion through the unstirred aqueous layers A_B and C_B is so rapid that it may be neglected in the subsequent discussion. We need only consider the slow diffusion in the non-aqueous layers B_o and B_i.

Partition Coefficients

Evidently the diffusion constants, D_K and D_{Na}, are too similar to account for the experimental fact that potassium enters C much faster than sodium. It would seem that the partition coefficients must be responsible for this difference.

In order to clarify the rôle of the partition coefficients let us first consider an hypothetical system in which KG and NaG are very weak electrolytes in A, B, and C, and in which C contains no CO_2. Let the values of K_o and Na_o be constant at 0.05, the values[12] of S_{K_o} and S_{K_i} being 0.62, those of S_{Na_o} and S_{Na_i} being 0.29, and those of D_K and D_{Na}

[9] This has been shown by a group of physical chemists whose results will shortly be published.

[10] Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, 1928–29, **12**, 543.

[11] The viscosity of water at 30.73°C. in C. G. S. units is 0.007905 and that of guaiacol at 30°C. is 0.0445. *Cf.* Landolt, H., and Börnstein, R., Physikalisch-chemische Tabellen, Berlin, Julius Springer, 5th edition (Roth, W. A., and Scheel, K.), 1923, **1**, 135, and 1931, suppl. vol. **2**, 102.

[12] These vary with concentration but we may assume for purposes of calculation that S_{KG_i} is constant as well as S_{KG_o}.

being equal. A simple calculation[13] indicates that in the early part of the experiment potassium should enter C faster than sodium and that $K_i \div Na_i$ should be less than $S_{K_o} \div S_{Na_o}$. The experiments bear this out. The calculation is as follows:

Let us suppose that the unstirred layers are so thin that the concentration gradient in B is approximately linear and that C is a mere film, only a few molecules thick, and contains at the start only distilled water, the bubbling of CO_2 being omitted. Let us now consider a layer in B of the same thickness as C adjoining the interface: this will be called $B_{i,}$. For convenience we suppose the total volume of each of these films to be 1 liter.

At the start of the experiment the concentration gradient of KG in B, which will be called G_K', is 0.031 − 0 = 0.031, and the corresponding value for G'_{Na} is 0.0145 − 0 = 0.0145. Hence $G_K' \div G_{Na}' = 2.14$. We may assume that during the first small increment of time 1.62 millimoles of KG reach $B_{i,}$ and distribute themselves so that 1.0 goes into C and 0.62 remains in $B_{i,}$, giving a concentration of 0.00062 M in $B_{i,}$ and 0.001 M in C (the value of K_o' remains constant at 0.031 since K_o is constant).

Since at the start G'_{Na} is 0.0145 and G'_K is 0.031 we suppose that the amount of NaG moving across B in the first small increment of time is approximately 1.62 (0.0145 ÷ 0.031) = 0.756 millimole: of this 0.17 will remain in $B_{i,}$ and 0.586 will pass into C (so that $S_{Na_i} = 0.17 \div 0.586 = 0.29$; this is the value previously stated). At the end of the first increment of time $G_K' = 0.031 - 0.00062 = 0.0304$ and $G_{Na}' = 0.0145 - 0.00017 = 0.0143$. Hence $G_K' \div G_{Na}' = 0.0304 \div 0.0143 = 2.1$ and $K_i \div Na_i = 0.001 \div 0.000586 = 1.7$.[14]

This calculation indicates that in the early part of the experiment the ratio $K_i \div Na_i$ will be greater than unity and that as time goes

[13] The significance of this method of calculation may seem doubtful, especially when we are not dealing with the earliest stages of the experiment, but it seems to be borne out by the results.

[14] It is of interest to note that the ratio $K_i \div Na_i$ in the early part of the experiment depends on the magnitudes of S_{KG_o} and S_{NaG_o} as well as on their ratio. Thus in the foregoing calculation we had $S_{KG_o} \div S_{NaG_o} = 0.62 \div 0.29 = 2.14$ and the value $K_i \div Na_i$ after the first increment of time was 1.7, but if we put $S_{KG_o} \div S_{NaG_o} = 6.2 \div 2.9 = 2.14$ we get a different result for $K_i \div Na_i$ since the value is 1.16. This may be shown as follows. Since G_K' and G_{Na}' are now ten times as great at the start we expect 16.2 millimoles of potassium and 7.56 of sodium to be moved. The concentrations in $B_{i,}$ are therefore KG = 0.0162 M and NaG = 0.00756 M. These will distribute so that $K_i' = 0.01395$ M, $K_i = 0.00225$ M, $Na_i' = 0.00562$ M, and $Na_i = 0.00194$ M. Hence $K_i \div Na_i = 0.00225 \div 0.00194 = 1.16$.

on it will approach unity since the concentrations in C will approach those in A (*i.e.* 0.05 M KG + 0.05 M NaG).

The actual model (with no CO_2 in C) differs from this hypothetical case since S_{K_i} is not constant and is not equal to S_{K_o}: this applies also to sodium. Furthermore KG and NaG are strong electrolytes in A and C[1] (though weak electrolytes in B) but this merely means that the diffusion of molecules of KG and NaG in C is replaced by the diffusion of the ions K^+, Na^+, and G^-, thereby changing somewhat the diffusion constants.

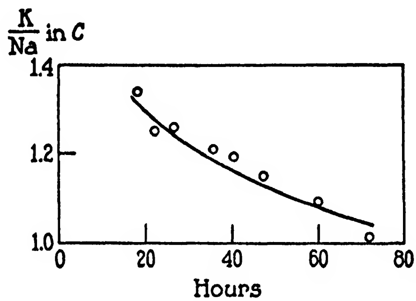

FIG. 2. Ratios of potassium to sodium in the artificial sap in C (Experiment 112). Model with 0.05 M KG + 0.05 M NaG in A, G. C. mixture in B, and distilled water in C (no CO_2). The ratio of partition coefficients is $S_{KG_o} \div S_{NaG_o} = 2.14$. Toward the end of the experiment the ratio approaches unity, as would be expected. In the first part of the curve the experimental errors are larger because the concentrations of sodium and potassium in C are relatively low.

Hence we expect that at the start potassium will enter C more rapidly than sodium and that both will reach the same concentration at equilibrium. This was found to be the case in earlier experiments (*e.g.* in Experiment 66 reported in a previous paper[15]). The ratios found in a later experiment (Experiment 112) are shown in Fig. 2 (in this, as in the other figures, the curves are drawn free-hand to give an approximate fit). In this case A contained 0.05 M KG + 0.05 M NaG and the ratio $S_{KG_o} \div S_{NaG_o}$ was 2.14 (see p. 208).

Model I was employed and a steady flow was maintained in A which kept its composition approximately constant: B contained G. C. mixture and C contained

[15] Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 667, Fig. 6.

distilled water (no CO_2 bubbling), stirred with a stream of air. The volume of C at the start was 150 cc. The temperature was 21° ± 2°C. The concentration of potassium + sodium in C was determined by titration with standard acid and of sodium by the method previously described,[2] potassium being determined by difference.

In most of our experiments CO_2 was present in C and in consequence KG and NaG reacted to form $KHCO_3$ and $NaHCO_3$ as soon as they reached the surface of C.[16] Potassium and sodium then diffused through C_B in the form of bicarbonates. But since diffusion in this layer is very rapid as compared with that in B it may be left out of account. (An additional effect of the reaction with CO_2 is a great reduction of the ionic activity products (K)(G) and (Na)(G) in C.)

Before discussing such experiments it is desirable to consider more fully the equation for penetration. In doing this we may neglect $D_K \div D_{Na}$ (since it is not far from unity) and write as a first approximation

$$\frac{R_K}{R_{Na}} = \frac{K'_o - K'_i}{Na'_o - Na'_i}$$

The values of K'_o and Na'_o are kept approximately constant by continually renewing the solution in A. It has been shown elsewhere[5] that the activity of K'_o is proportional to the ionic activity product[17] $(K_o)(OH_o)$ in A. Hence we may put $K'_o = S_{K_o}K_o = C_4(K_o)(OH_o)$ and it is evident that the value of S_{K_i} will depend on that of OH^-. We may treat K'_i in the same way. All of this applies equally to sodium.

As already stated $K'_o = S_{K_o}K_o$: hence to approximate the value of K'_o a determination of S_{K_o} = (concentration of undissociated KG in

[16] This might not have much effect on the value of $K_i \div Na_i$ as calculated by the method given on p. 205, since it might influence both of the diffusing substances in similar fashion.

[17] Under the conditions of the experiment the activity of OH in C bears a constant relation to the activity of HCO_3^- and to that of the guaiacol ion G^-.

We may write $K'_o = C_3(K_o)(OH_o)$ and $K'_i = C_4(K_i)(OH_i)$ where the subscripts o and i refer to the outside and inside solutions respectively. But C_3 and C_4 vary with concentration because the non-aqueous phase changes.

the surface of B_o) $\div$ (concentration of K^+ in A) is desirable. This cannot be done directly but we may approximate it by determining[18] S_{KG_o} = (concentration of KG in the surface of B_o) $\div$ (concentration of K^+ in A). It seems highly probable that the ratio $S_{K_o} \div S_{Na_o}$ is approximately equal to $S_{KG_o} \div S_{NaG_o}$ and since we need comparative values only we may use S_{KG_o} and S_{NaG_o} in place of S_{K_o} and S_{Na_o}.

The values of S_{KG} and S_{NaG} were determined as follows. An aqueous solution containing equal concentrations of KG and NaG was shaken with a relatively small volume of G. C. mixture on a shaking machine and was then allowed to stand for 24 hours or longer. The G. C. mixture was then shaken with half its volume of 0.08 M HCl which removed practically all of the potassium and sodium. The determinations of potassium and of sodium were made as previously described.[2] The temperature was 21° ± 2°C.

Since the aqueous phase loses more potassium than sodium it must be relatively large to keep its ratio $K_o \div Na_o$ approximately constant or else it must contain more potassium at the start. As an illustration of the latter method we may cite the following: 104 cc. of aqueous solution of 0.222 M KOH + 0.151 M NaOH was shaken with 100 cc. G. C. mixture. After separation there was 98 cc. of the aqueous solution in which the concentrations were 0.107 M potassium and 0.110 M sodium. There was also 106 cc. G. C. mixture in which the concentrations were 0.116 M potassium and 0.053 M sodium. Hence we have $S_{KG_o} = 0.116 \div 0.107 = 1.085$ and $S_{NaG_o} = 0.053 \div 0.110 = 0.482$. Hence $S_{KG_o} \div S_{NaG_o} = 1.085 \div 0.482 = 2.26$.

The situation is easily seen from the following considerations. For the aqueous phase let us put: volume = V, concentration of potassium = C_K, concentration of sodium = C_{Na}, moles of potassium = M_K, moles of sodium = M_{Na}, and designate the corresponding values in the non-aqueous mixture as V', C_K', etc. Assuming that $V = 1$ liter, $V' = 10$ liters, and that owing to the nature of the non-aqueous phase the partition coefficient, *i.e.* $C_K' \div C_K$ has a value of 0.4, and that at the start $C_K = C_{Na} = 1$, we have at equilibrium (providing no change in volume occurs and that potassium and sodium act independently)

$$C_K = M_K = 1 - M_K'$$

$$\frac{M_K'}{10} = C_K' = 0.4C_K = 0.4(1 - M_K')$$

[18] Strictly speaking we do not determine undissociated KG in B_o but total (stoichiometric) concentration of potassium in B_o which amounts to practically the same thing since the concentrations of KOH and $KHCO_3$ in B_o are negligible in comparison with that of KG, and KG is a very weak electrolyte in B (as shown by the unpublished work of physical chemists).

hence

$$M_K = 0.8$$
$$C_K = M_K = 1.0 - 0.8 = 0.2$$
$$C'_K = 0.8 \div 10 = 0.08$$

Hence $C'_K \div C_K = 0.08 \div 0.2 = 0.4$: this is the value previously assumed for the partition coefficient.

Assuming that the partition coefficient for sodium, *i.e.* $C_{Na}' \div C_{Na} = 0.2$ and proceeding in the same way we obtain for equilibrium $C_{Na} = 0.335$, $C_K \div C_{Na} = 0.2 \div 0.335 = 0.594$; also $C_{Na}' = 0.067$ and $C_K' \div C_{Na}' = 0.08 \div 0.067 = 1.2$. This latter value changes to 1.02 when we put $V = 1$ and $V' = 100$ (instead of $V' = 10$), to 1.71 when we put $V = 1$ and $V' = 1$, and to 1.95 when we put $V = 1$ and $V' = 0.1$.

We see that the greater the relative volume of the aqueous solution the nearer the ratio $C'_K \div C'_{Na}$ in the non-aqueous phase approaches to the ratio of partition coefficients which in this case is $0.4 \div 0.2 = 2$.

A series of values of S_{KG_o} and S_{NaG_o} is shown in Fig. 3. They were determined when both K*G* and Na*G* were present in equal concentrations in the aqueous phase at equilibrium.[19]

Let us now consider conditions at the inner phase boundary. Here the partition coefficients are S_{K_i} and S_{Na_i} (where $S_{K_i} = K'_i \div K_i$ and $S_{Na_i} = Na'_i \div Na_i$) We may use S_{KG_i} = (concentration of K*G* in the surface of B_i) ÷ (concentration of K^+ in *C*) in place of S_{K_i}.

The determinations are somewhat uncertain because on reaching *C*, K*G* is transformed to $KHCO_3$[5], and we must therefore determine S_{KG_i} by shaking up an aqueous solution of $KHCO_3$ with G. C. mixture so that not only K*G* but also $KHCO_3$ is taken up. We can determine the total amount of potassium taken up but we do not know exactly what percentage of this is K*G*.[20] We can also deter-

[19] Adding Na*G* to K*G* affects S_{KG_o} as follows: As stated elsewhere (footnote 5) K'_o is proportional to the ionic product $(K_o)(G_o)$ when activities are taken. Using concentrations (and taking total potassium in *B* as approximately equal to K'_o) we may say that since S_{KG_o} equals (K*G* in *B*) ÷ (potassium in *A*) the value of K*G* in *B* will be approximately doubled when we double G_o by adding Na*G*: this will double the value of S_{KG_o} since the concentration of potassium in *A* remains constant. This has been tested experimentally and is found to be approximately true.

[20] For example, when 0.63 M $KHCO_3$ at pH 7.5 was shaken up with G. C. mixture the concentration of potassium in the latter was 0.0062 M. The concentration of $CO_2 + HCO_3$ was 0.0025 M. Probably most of this was CO_2: hence if we put $CO_2 = 0.002$ M we have K*G* = 0.0062 − 0.0005 = 0.0057 M. (It depends on the pH value of the aqueous solution.) *Cf.* footnote 5.

mine the total carbon taken up but we do not know exactly what per cent of this is HCO_3 and what is CO_2. *We find that (concentration of potassium in G. C. mixture) ÷ (concentration of potassium in aqueous phase) varies with concentration and hence we conclude that S_{K_i} varies with concentration.* This also applies to S_{Na_i}.

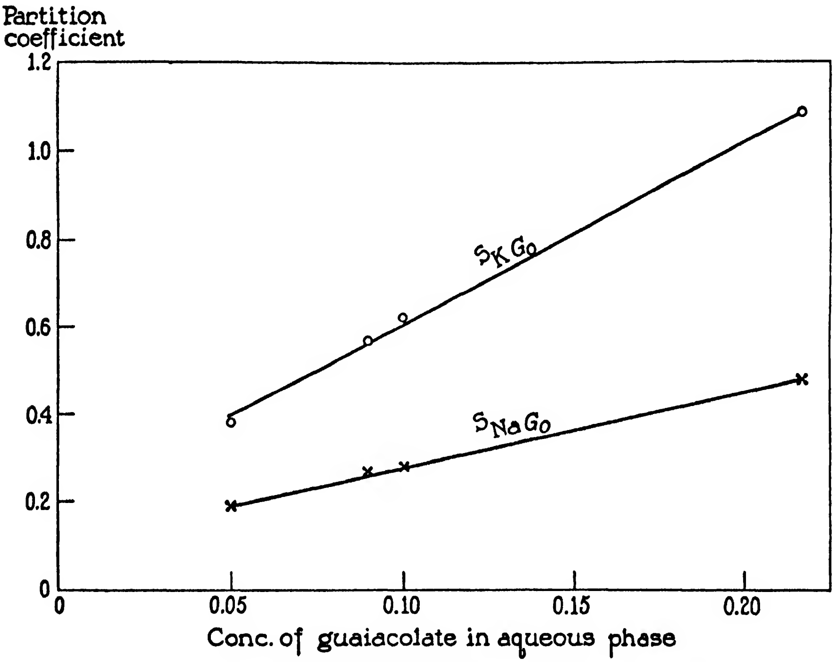

FIG. 3. Values of the partition coefficients S_{KG_o} and S_{NaG_o}. These values were determined in the presence of equal concentrations of potassium and sodium. The total concentration of G^-, or guaiacol ion (taken as equal to $KG + NaG$), is plotted as abscissae. Thus the value for 0.05 means that an aqueous solution of 0.025 M KG + 0.025 M NaG was shaken with a relatively small volume of G. C. mixture and the partition coefficients were calculated from the amounts of KG and NaG found in the G. C. mixture.

Since the ratio $S_{K_i} \div S_{Na_i}$ varies with concentration (independently of pH) and since the concentration of $K_i + Na_i$ may rise during the experiment from zero to 1.2 M there is opportunity for a change in the ratio $K'_i \div Na'_i$ which will affect the relative rates of entrance of potassium and sodium.

The values of S_{K_i} and S_{Na_i} also vary with pH (p. 207). Since the

pH value in C may rise[2] from between 5 and 6 to the neighborhood of 7.6 during an experiment the values of S_{K_i} and S_{Na_i} must increase accordingly. Variation in the flow of CO_2 will also affect the pH.

It should be remembered that the diffusion "constants" D_K and D_{Na} as here used are not constant since D_K varies with the concentration of NaG and *vice versa* (p. 203).

Other Factors

It thus appears that penetration depends largely on the diffusion constants and partition coefficients. But it is also affected by a number of other factors, among which are the following:

1. Even when the concentrations in the main body of A are kept constant[2] those at the interface may vary for there is a concentration gradient in the unstirred layer A_B which determines the concentration at the outer surface of B. The more rapid the stirring the thinner this layer and consequently the nearer K_o and Na_o (*i.e.* the concentrations of K^+ and Na^+ at the inner surface of A_B) will approach the concentrations in the main body of A. When the concentrations in A are altered the value of $K'_o \div Na'_o$ may vary because the partition coefficients change. The ratio $K'_o \div Na'_o$ (and therefore of $R_K \div R_{Na}$) will therefore depend in part on the rate of stirring.

This ratio may also be influenced by temperature which alters the viscosity and hence the thickness of the layers (the viscosity is altered by the presence of electrolytes). Temperature may also affect the partition coefficients unequally.

If a model be used with diffusion in B more rapid than in A the ratio $K_o \div Na_o$ may vary because, with potassium moving into B_o more rapidly than sodium, its relative concentration in A_B must become steadily less, unless the rates of stirring and of diffusion be sufficient to renew the supply (see p. 208). As K_o decreases potassium will move more slowly into B.

2. In the stirred layers the forward movement of electrolyte will depend on such factors as rate of stirring, and viscosity.

3. There are concentration gradients in C_B which will depend on the factors already enumerated and which may change the ratio $R_K \div R_{Na}$.

4. Surface forces may play a part; *e.g.*, substances diminishing surface tension will tend to remain in the surface.

5. Reactions may occur at the outer surface and if slow enough may affect the result; *e.g.*, loss of water by the penetrating substance or molecular association in the non-aqueous phase. At the inner surface the reverse processes will occur but here we may have in addition combinations with acids or other substances in C.

6. The penetration of electrolytes into C is accompanied by that of water. To understand its effect let us consider what happens when water is added suddenly[21] to C. Thus if the concentration gradients in B be called G'_K and G'_{Na} and the ratio be

$$\frac{G'_K}{G'_{Na}} = \frac{K'_o - K'_i}{Na'_o - Na'_i} = \frac{0.24 - 0.12}{0.10 - 0.04} = 2$$

and water be added to C, doubling its volume, we have

$$\frac{G'_K}{G'_{Na}} = \frac{0.24 - 0.06}{0.10 - 0.02} = 2.25$$

In this case where the ratio $K'_o \div K'_i = 2$ is less than $Na'_o \div Na'_i = 2.5$ we observe that the ratio $G'_K \div G'_{Na}$ rises after the addition of water.

When $K'_o \div K'_i$ is greater than $Na'_o \div Na'_i$ the ratio falls. Thus if $K'_o \div K'_i = 0.24 \div 0.02 = 12$ and $Na'_o \div Na'_i = 0.13 \div 0.02 = 6.5$ we have before the addition of water

$$\frac{G'_K}{G'_{Na}} = \frac{0.24 - 0.02}{0.13 - 0.02} = 2$$

and afterwards

$$\frac{G'_K}{G'_{Na}} = \frac{0.24 - 0.01}{0.13 - 0.01} = 1.92$$

When $K_o \div K_i = Na_o \div Na_i$ the addition of water does not change the ratio. Thus if $K'_o \div K'_i = 0.24 \div 0.12 = 2$ and $Na'_o \div Na'_i = 0.08 \div 0.04 = 2$ we have before the addition of water

[21] After the sudden addition of water the concentration gradients would no longer be linear.

$$\frac{G'_K}{G'_{Na}} = \frac{0.24 - 0.12}{0.08 - 0.04} = 3$$

and afterwards

$$\frac{G'_K}{G'_{Na}} = \frac{0.24 - 0.06}{0.08 - 0.02} = 3$$

Evaporation will, of course, produce the opposite effect.

The inward movement of water may have a different temperature coefficient from the movement of the penetrating substances: hence the composition of *C* may depend on temperature.[22]

7. There is an outward movement of substances (from *C* to *A*); *e.g.*, of $KHCO_3$, $NaHCO_3$, CO_2, and H_2CO_3. This may be neglected in the earlier part of the process of penetration but toward the end and in the steady state it may become more important. It is quite possible that the outward movement of $KHCO_3$ may differ from that of $NaHCO_3$ in such fashion as to produce a different ratio of $K_i \div Na_i$ from that which would otherwise occur.

In spite of the fact that penetration is influenced by so many factors the experiments indicate that the ratio of potassium to sodium in *C* depends chiefly on the partition coefficients.

EXPERIMENTS

(*a*) *Diffusion from A to C*

To illustrate this statement we may cite a series of experiments, in which *A* contained equal concentrations of K*G* and Na*G*, *B* contained G. C. mixture, and *C* contained distilled water in which CO_2 was bubbling. The results are shown in Table I (p. 214). It is evident that potassium predominates in *C* in every case as would be expected in view of its higher partition coefficient. We see also that the average of the ratio K ÷ Na in *C* does not differ greatly from $S_{KG_o} \div S_{NaG_o}$. There is considerable variation in the ratios of K ÷ Na: changes during the progress of one experiment are shown in Fig. 4.

Models I, II, and III were used:[2] a steady flow was maintained in *A* thus keeping its composition approximately constant. *A*, *B*, and *C* were stirred. The temperature varied between 20 and 25°C. The determinations of potassium and sodium were made as previously described.[2]

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[22] At the start there may be an outward movement of water since the concentration of electrolytes is greater in *A* than in *B*.

TABLE I

Ratios of Potassium to Sodium in the Artificial Sap in C

Experiment	Time of penetration	Ratio K ÷ Na in C		Solution in A	Ratio of partition coefficients $S_{KG_o} \div S_{NaG_o}$
	days				
109 *a*	10	2.45	Not in steady state	0.02 M K*G* + 0.02 M Na*G*	2.1
b	8	2.37			
c	11	2.28			
58	9	1.4		0.1 M K*G* + 0.1 M Na*G*	2.3
63	12	1.5			
		Av. = 2.0			
66	33	1.6	In steady state	0.05 M K*G* + 0.05 M Na*G*	2.14
80	4	2.7			
81	4	2.7			
111	50	2.0			
		Av. = 2.3			

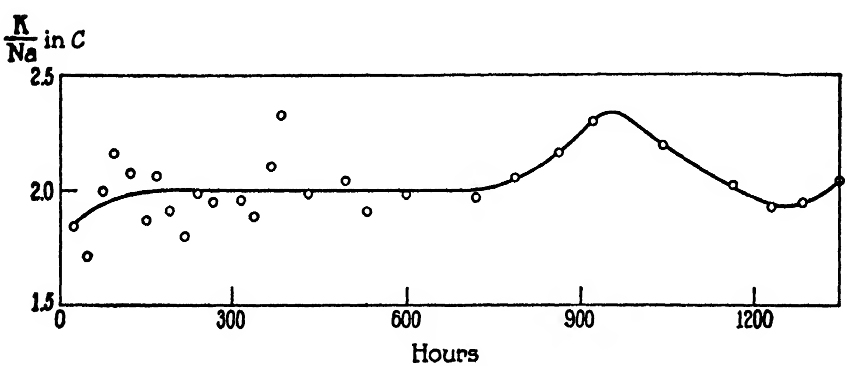

FIG. 4. Ratios of potassium ÷ sodium in C in a model in which A contained 0.05 M KG + 0.05 M NaG: B contained G. C. mixture, and C contained distilled water at the start but had CO_2 bubbling through it throughout the experiment (Experiment 111). The ratio of partition coefficients is $S_{KG_o} \div S_{NaG_o} = 2.14$. The experimental errors are larger during the first part of the experiment when the concentrations in C are low.

(b) *Diffusion from A to B*

It seemed desirable to examine the process of penetration at each interface separately. To examine the outer interface a model was constructed with A and B but without C. In A was placed 0.1 M KG + 0.1 M NaG, and in B was placed G. C. mixture. The results of a typical experiment (No. 83) are shown in Fig. 5.

In this case the concentration gradient of chief importance is that in the layer B_o. Calling the concentration of undissociated KG at the outer surface of this layer K'_o and that at the inner limit[23] of this layer

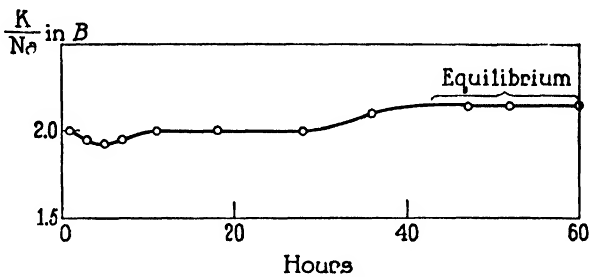

FIG. 5. Ratios of potassium ÷ sodium in B in Experiment 83 dealing with the interface between A and B (C was omitted from this model). The ratio of partition coefficients is $S_{KG_o} \div S_{NaG_o} = 2.3$. The experimental errors are larger during the first part of the experiment when the concentrations in C are low.

(f, Fig. 1) K'_f (with similar designations for sodium) we may write as an approximation

$$\frac{R_K}{R_{Na}} = \frac{K'_o - K'_f}{Na'_o - Na'_f} = \frac{(S_{KG_o}K_o) - K'_f}{(S_{NaG_o}Na_o) - Na'_f}$$

At the start, when K'_f and Na'_f are both equal to zero, we may write as an approximation

$$\frac{R_K}{R_{Na}} = \frac{S_{KG_o}K_o}{S_{NaG_o}Na_o}$$

Since $K_o = Na_o$ we have

$$\frac{R_K}{R_{Na}} = \frac{S_{KG_o}}{S_{NaG_o}}$$

[23] This is merely a convenient fiction since there is no definite limit at this spot.

We therefore expect at the start a correlation between the partition coefficients and the concentrations of potassium and sodium in B. This will also be true as the system approaches equilibrium. Hence it may well be true of the intermediate period and this appears to be the case since the ratio of $S_{KG_o} \div S_{NaG_o}$ was found in shaking experiments (p. 208) to be $1.085 \div 0.48 = 2.26$ which is fairly close to the ratios observed during the progress of the experiment as shown in Fig. 5.

When approximate equilibrium was reached in this experiment the concentration of potassium in B was 0.126 M and that of sodium 0.055 M. Hence we have $S_{KG_o} = 1.26$ and $S_{NaG_o} = 0.55$: these values are a little higher than those obtained in the shaking experiments[24] (where we found $S_{KG_o} = 1.085$ and $S_{NaG_o} = 0.48$. The ratio $S_{KG_o} \div S_{NaG_o} = 2.29$ is also a little higher than the value of 2.26 found in shaking experiments.

Model III was employed.[2] Both A and B were stirred. A continuous flow in A kept the composition constant to within 5 per cent. B contained 500 cc. of G. C. mixture. Equilibrium was attained in about 52 hours. The temperature was 20° ± 2°C.

(c) *Diffusion from B to C*

In order to examine the processes occurring at the interface between B and C a model was used in which B was brought into equilibrium with A (by shaking B with a great excess of A) before the experiment was started (so that there was relatively little diffusion from A to B during the experiment): C contained only distilled water at the start (no CO_2 was bubbled during the experiment). In this case the chief movement during the early part of the experiment was across the interface between B and C so that the layer of chief importance is B_i. Calling the concentration of undissociated KG at the outer limit[25] (*g*, Fig. 1) of this layer K'_o and that at the other surface K'_i (with similar designations for sodium) we may write as an approximation

[24] *I.e.* experiments in which a solution containing 0.107 M KOH + 0.110 M NaOH was shaken up with G. C. mixture as described on p. 208. As shown in Fig. 3 the ratio $S_{KG_o} \div S_{NaG_o}$ is very nearly the same at a concentration of 0.2 M G^- as at a concentration of 0.217 M.

[25] This is, of course, a convenient fiction as there is no sharp limit at this spot.

$$\frac{R_K}{R_{Na}} = \frac{K'_o - K'_i}{Na'_o - Na'_i}$$

A typical experiment (Experiment 86) was started with 0.1 M KG + 0.1 M NaG in A, distilled water saturated with guaiacol (no CO_2 bubbling) in C, and with B in equilibrium with A. From the very start B contained 0.1085 M KG and 0.048 M NaG. Since S_{KG_o} = 1.085 and S_{NaG_o} = 0.48 the ratio $S_{KG_o} \div S_{NaG_o}$ is 2.26. A simple calculation shows that soon after the start of the experiment $K_i \div Na_i$ might be in the neighborhood of 1.6 and this agrees fairly well (Fig. 6) with observa-

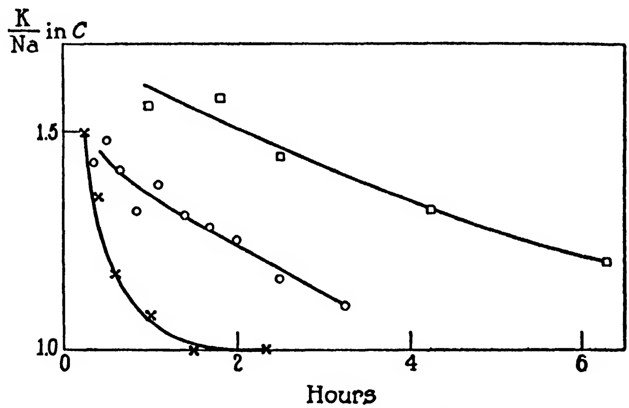

FIG. 6. Ratios of potassium ÷ sodium in C in Experiments 86 *a* (×), 86 *b* (○), and 86 *c* (□), dealing with the interface between B and C. The ratio of partition coefficients is $S_{KG_o} \div S_{NaG_o}$ = 2.3. The experimental errors are larger during the first part of the experiment when the concentrations in C are low.

tion (although the validity of this method of calculation may be questioned: see p. 205). As $K_o = Na_o$ we expect that as equilibrium is approached $K_i \div Na_i$ will approach unity and this appears to be the case. The calculation is as follows:

Consider a thin layer of B, only a few molecules thick, adjoining the inner interface: we may call this B_{i}, and the corresponding layer in C on the other side of the interface C_{Bs}. For convenience we put the volume of each of these layers at 1 liter.

We see that B_{i}, contains 0.1085 mole of KG and we may suppose that when water is brought in contact with it enough KG instantaneously moves into C_{Bs} to bring these two layers into approximate equilibrium. If during this process no more

KG moves into B_i, we may make the following calculation: After approximate equilibrium is reached we have

$$\frac{K_i' = 0.1085 - K_i}{K_i} = S_{KG_i}$$

If for convenience[26] we put $S_{KG_i} = S_{KG_o} = 1.085$ we have

$$\frac{0.1085 - K_i}{K_i} = 1.085$$

whence $K_i = 0.052$ and $K_i' = 0.0565$. In the same way we have

$$\frac{0.048 - Na_i}{Na_i} = 0.48$$

whence $Na_i = 0.0324$ and $Na'_i = 0.0156$. Hence $K_i \div Na_i = 0.052 \div 0.0324 = 1.6$. We then have for the ratio of gradients

$$\frac{K_o' - K_i'}{Na'_o - Na'_i} = \frac{0.1085 - 0.0565}{0.048 - 0.0156} = 1.6$$

so that we might expect $R_K \div R_{Na}$ to be about 1.6 as indeed appears to be the case with the upper curve in Fig. 6 extrapolated to zero time.

Model III was employed.[2] A constant flow in A kept its composition nearly constant: B was brought into equilibrium with A by shaking the two phases together before the experiment started: C contained at the start 75 cc. of distilled water saturated with G. C. mixture and was stirred by a stream of air. A and B were stirred mechanically. The temperature was 20° ± 2°C.

In another sort of experiment[27] (Experiment 66) no CO_2 was bubbled during the first 236 hours and the concentrations in C reached the same level as in A, namely 0.05 M KG + 0.05 M NaG: B was then practically uniform throughout and contained 0.031 M KG + 0.0145 M NaG. The ratio $S_{KG_o} \div S_{NaG_o}$ was $0.62 \div 0.29 = 2.14$.

At 236 hours the bubbling of CO_2 began and 4 hours later the ratio K ÷ Na in C was 1.65 (as shown at the ordinate marked 240 in Fig. 7). The ratio rose until the 264th hour after which it slowly fell and then gradually rose again.

[26] This makes the value of S_{KG_i} too large but as that of S_{NaG_i} will be too large by a corresponding amount the error in the ratios $K'_i \div Na'_i$ and $K_i \div Na_i$ will be small.

[27] Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 676, 678 (Experiment 66, Table I, and Fig. 6).

In the steady state[28] C contained 0.73 M $KHCO_3$ + 0.45 M $NaHCO_3$, giving a ratio of 1.62.

Model I was employed. A, B, and C were stirred as usual. A constant flow was maintained in A. The temperature varied from 20 to 25°C. during the course of the experiment.

In concluding the experimental part we may say that in all cases (the diffusion constants being nearly equal) the ratio K ÷ Na in C seems to depend chiefly on the partition coefficients. But other

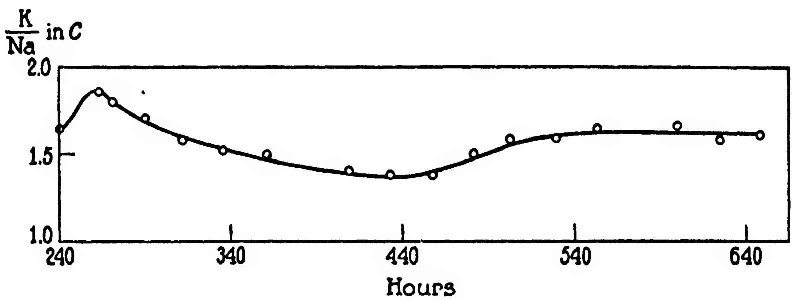

FIG. 7. Ratio of potassium ÷ sodium in C in Experiment 66, dealing with the interface between B and C. During the first part of the experiment no CO_2 was bubbled through C. At 236 hours the ratio in C was unity and B was in equilibrium with A. The bubbling of CO_2 was then commenced and the ratio quickly changed: at 240 hours it was 1.65. The ratio of partition coefficients was $S_{KG_o} \div S_{NaG_o} = 2.2$. In the first part of the curve there are larger experimental errors in determining the concentrations of sodium and potassium (since they are relatively low).

factors are sufficiently influential to produce considerable variation in these ratios.

DISCUSSION

It seems probable that many of the variables discussed in this paper are found in living cells such as those of *Nitella* and of *Valonia*.[29]

[28] At this time A contained 0.043 M KG + 0.007 M $KHCO_3$ + 0.043 M NaG + 0.007 M $NaHCO_3$. The bicarbonate was due to diffusion of HCO_3^- and CO_2 from C into A.

[29] The models would resemble the living cells more closely if we employed KOH and NaOH in A and if KG and NaG, though soluble in the non-aqueous layer, were practically insoluble in water.

In *Valonia* and *Nitella* we apparently have a continuous non-aqueous phase at the inner and outer protoplasmic surfaces.[30] *We may suppose that these surface layers correspond to the layers B_o and B_i in the model and that between them is an aqueous phase more or less stirred by protoplasmic movement or by convection currents. The external solution and the sap are well stirred by convection currents. We therefore seem to have opportunity for some of the variables that are found in models.

In these models the chief factors appear to be partition coefficients and diffusion constants. Is this true of living cells in general?

In seeking to answer this question we must remember that the importance of the partition coefficients depends on the speed of diffusion in the non-aqueous layers. When diffusion in these layers is slow enough to control penetration the partition coefficients become important. It would seem that this applies to most living cells since partition coefficients appear to play a highly important rôle. It is for this reason that Overton's theory is so useful, especially as amended by Irwin.

When the partition coefficients of two substances are not very different molecular size becomes important because it determines the diffusion constants. It may also be due in some cases to the fact that penetration is regulated more by the cell wall than by the protoplasm.

In many cases the non-aqueous layers are probably very thin. Hence the diffusion constants in these layers must be very small or the concentration gradients (due to the partition coefficients at the two surfaces of the layer) must be very gentle in order to make diffusion slow enough to have the process of penetration controlled by this layer.

The diffusion constants would, of course, be small if the viscosity were high. It is not necessary to suppose that the layers are solid since protoplasm in contact with water rounds up as though true surface tension existed.[30]

It may be remarked in passing that the idea that protoplasm has a non-aqueous surface has been opposed on the ground that water and salts enter freely. But the model shows that this objection does not hold since water and salts freely pass through the non-aqueous layer.

[30] Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, **35**, 967.

Let us now consider the question of ionization. In our models K*G* penetrates very rapidly as compared with KCl although both are equally ionized in the external solution. But both are weak electrolytes in the non-aqueous phase and hence penetrate chiefly in molecular form. The difference lies in the fact that K*G* has a much higher partition coefficient than KCl.

There is another consideration which may well be mentioned here, namely, that partition coefficients are important in bioelectric effects. Assuming the latter to be chiefly due to diffusion potentials[31] we may illustrate the situation by means of models. For example, when only K*G* is present the diffusion potential at the outer surface will depend on K'_o and at the inner surface on K'_i. Now when $S_{K_o} = S_{K_i}$ and $K_o \div K_i = 10$ then $K'_o \div K'_i = 10$. But when $S_{K_o} = 1$ and $S_{K_i} = 0.1$, and $K_o \div K_i = 10$ we have $K'_o \div K'_i = 100$ and the diffusion potential increases accordingly. Similar considerations would apply if K*G* were placed at the outer surface and Na*G* at the inner since the concentrations and consequently the potential in the non-aqueous phase would depend on the values of S_{K_o} and S_{Na_i}.

Aside from the question of partition coefficients the outstanding fact brought out by these experiments is the large number of variables concerned. No attempt has been made to treat all of these quantitatively or to set up equations for the time curve of penetration. In a previous paper an empirical equation was given which fits the observations satisfactorily when potassium alone is present and a rigorous treatment has been formulated by L. G. Longsworth[32] by means of which the time curve has been calculated. By means of other equations he has calculated the ratio of sodium to potassium in the steady state. But no attempt has been made as yet to include in this treatment all the variables mentioned in this paper.

The fact that with constant concentrations of potassium and sodium outside there is so much variation in their proportions in the artificial sap in *C* recalls the situation in *Valonia* where there is a considerable variation in the cell sap. It happens that the variation is similar in the two cases for if we divide the highest observed ratio of K ÷ Na by

[31] Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 715; *Biol. Rev.*, 1931, **6**, 369; *Ergebn. Physiol.*, 1933, **35**, 967.

[32] Longsworth, L. G., *J. Gen. Physiol.*, 1933–34, **17**, 211.

the lowest we get in the model $2.7 \div 1.4 = 1.93$ and in the case of *Valonia*[1] $5.72 \div 2.55 = 2.24$.

In conclusion it may be appropriate to repeat that when the living cell shows a great difference in the penetration of similar compounds of potassium and of sodium it seems safe to conclude that diffusion coefficients cannot be responsible since the molecular sizes cannot differ greatly. Hence the difference must lie in partition coefficients. This is a very important factor in dealing with living organisms.

SUMMARY

Some of the factors affecting penetration in living cells may be advantageously studied in models in which the organic salts *KG* and *NaG* diffuse from an aqueous solution *A*, through a non-aqueous layer *B* (representing the protoplasmic surface) into an aqueous solution *C* (representing the sap and hence called artificial sap) where they react with CO_2 to form $KHCO_3$ and $NaHCO_3$. Their relative proportions in *C* depend chiefly on the partition coefficients and on the diffusion constants in the non-aqueous layer. But the ratio is also affected by other variables, among which are the following:

1. Temperature, affecting diffusion constants and partition coefficients and altering the thickness of the unstirred layers by changing viscosity.
2. Viscosity (especially in the non-aqueous layers) which depends on temperature and the presence of solutes.
3. Rate of stirring, which affects the thickness of the unstirred layers and the transport of electrolyte in those that are stirred.
4. Shape and surface area of the non-aqueous layer.
5. Surface forces.
6. Reactions occurring at the outer surface such as loss of water by the electrolyte or its molecular association in the non-aqueous phase. The reverse processes will occur at the inner surface and here also combinations with acids or other substances in the "artificial sap" may occur.
7. Outward diffusion from the artificial sap. The outward movement of $KHCO_3$ and $NaHCO_3$ is small compared with the inward movement of *KG* and *NaG* when the concentrations are equal. This

is because the partition coefficients[8] of the bicarbonates are very low as compared with those of Na*G* and K*G*.

Since CO_2 and HCO_3^- diffuse into *A* and combine with K*G* and Na*G* the inward movement of potassium and sodium falls off in proportion as the concentration of K*G* and Na*G* is lessened.

8. Movement of water into the non-aqueous phase and into the artificial sap. This may have a higher temperature coefficient than the penetration of electrolytes.

9. Variation of the partition coefficients with concentration and pH.

Many of these variables may occur in living cells. (It happens that the range of variation in the ratio of potassium to sodium in the models resembles that found in *Valonia*.)

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THE KINETICS OF PENETRATION

VII. Molecular versus Ionic Transport

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In many living cells potassium penetrates more rapidly than sodium. Since the potassium ion has a greater mobility in water than the sodium ion some investigators see in this situation evidence that electrolytes pass chiefly as ions through the protoplasmic surface, in which the order of ionic mobilities is supposed to be that found in water.

If it could be demonstrated that the ionic mobilities correspond in this way[1] it would not show that electrolytes pass chiefly as ions through the protoplasmic surface for such a correspondence might equally well exist if the movement were mostly in molecular form.

This can be seen in certain models in which the protoplasmic surface[2] is represented by a non-aqueous layer B, and electrolytes pass[3] from an aqueous phase A through B into an aqueous phase C (representing the cell sap).

Let us consider a typical experiment in which A contained 0.05 M potassium plus 0.05 M sodium, both combined with guaiacol and *p*-

[1] Such a correspondence would be expected on the basis of Walden's rule according to which the mobility of an ion is inversely proportional to the viscosity of the medium. Hence the order of mobilities would be the same in all media. This rule seems to work much better for large ions than for small ones (*cf.* Ulich, H., *Tr. Faraday Soc.*, 1927, **23**, 388). On this basis we should expect the ratio of U_K (the mobility of the potassium ion) divided by U_{Na} (the mobility of the sodium ion) to be constant in all media but this may not be the case. In the protoplasmic surface this ratio appears to be very much greater than in water (*cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13**, 715; *Ergebn. Physiol.*, 1933, **35**, 967) if we neglect partition coefficients and phase boundary potentials.

Evidently the result will depend greatly on the degree of solvation and on the formation of complex ions.

[2] Osterhout, W. J. V., *Biol. Rev.*, 1931, **6**, 369.

[3] Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 667.

cresol to form organic salts. For convenience the potassium salts will be lumped together and called K*G* (as in previous papers[3,4]). The sodium salts will be called Na*G*.

In *B* was a mixture of 70 per cent guaiacol + 30 per cent *p*-cresol which will be called G.C. mixture. *C* contained at the start distilled water and CO_2 was bubbled through it during the entire experiment.[5]

Under these conditions K*G* penetrated through *B* into *C* where it was transformed to $KHCO_3$ which does not readily pass out; Na*G* acted similarly. As a result potassium and sodium attained much higher concentrations in *C* than in *A*. Eventually a steady state was reached in which the volume of *C* increased while the composition remained approximately constant: the concentration of potassium in *C* in the steady state was about twice that of sodium.

All three phases were stirred but at the phase boundaries there were unstirred layers[3] in which penetration was slow because it depended on diffusion: in the unstirred non-aqueous layers, which may be called B_o and B_i (where the subscripts *o* and *i* refer to the outer and inner surfaces respectively) it was so much slower than elsewhere that these two layers controlled the process of penetration.

The unpublished work of physical chemists shows that K*G* and Na*G* are very weak electrolytes in *B* and we may therefore conclude that these salts move through *B* chiefly in molecular form.

Following the usage of previous papers[3,4,5] we may write as an approximation

$$\frac{R_K}{R_{Na}} = \left(\frac{D_K}{D_{Na}}\right) \frac{K'_o - K'_i}{Na'_o - Na'_i}$$

where R_K is the rate of entrance (in moles) of potassium into *C*, K'_i and K'_o are the concentrations of undissociated K*G* in the outer surface of B_o and the inner surface of B_i respectively, and D_K is the diffusion constant of undissociated K*G* in *B* (a corresponding nomenclature is used for Na*G*).[6]

[4] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529.

[5] This is Experiment 111. See Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933–34, **17**, 445.

[6] In this equation the dissociation of K*G* and Na*G* in *B* is neglected since it is small and since the values are used only for comparative purposes; *e.g.*, comparison of $K'_o - K'_i$ with $Na'_o - Na'_i$. It should be noted that this formula takes no account of the movement of water.

This may be rewritten

$$\frac{R_K}{R_{Na}} = \left(\frac{D_K}{D_{Na}}\right) \frac{K_o S_{K_o} - K_i S_{K_i}}{Na_o S_{Na_o} - Na_i S_{Na_i}}$$

where K_o is the concentration of potassium in the inner surface of *A*, K_i is the corresponding concentration in the outer surface of *C*: S_{K_o} and S_{K_i} are partition coefficients; *i.e.*, $S_{K_o} = K'_o \div K_o$ and $S_{K_i} = K'_i \div K_i$. A corresponding notation is used for Na*G*.

Since $D_K \div D_{Na}$ is nearly unity[6] it may be neglected and we may therefore suppose that the relative rate of penetration depends chiefly on the partition coefficients. We should therefore say that potassium predominates over sodium in *C* because the partition coefficient of K*G* is higher than that of Na*G*.

It is known from the unpublished investigations of physical chemists that the potassium ion has a higher mobility in *B* than the sodium ion. But it seems unlikely that this plays an important rôle since (*a*) the mobility of K^+ is only slightly greater than that of Na^+ and (*b*) both K*G* and Na*G* are very weak electrolytes in *B*.[5] Hence in passing through *B* both K*G* and Na*G* must move chiefly in molecular form and the rôle of ionic mobility is negligible.

In order therefore to explain the more rapid penetration of potassium we must suppose that the chief cause lies in the fact that the partition coefficient of K*G* is larger than that of Na*G* (Table I, p. 233) and in consequence the concentration gradient in *B* is greater.

We may now ask whether this applies to other substances. In order to test this we have determined the diffusion constants and the rates of penetration of the following pairs of organic alkali salts diffusing together: lithium and potassium; sodium and potassium; sodium and rubidium; and sodium and cesium.

The Northrop diffusion apparatus[7] was used with all the suggested precautions. The temperature was 25° ± 0.1°C.

The alkali G.C. salts were prepared by shaking an aqueous solution of the hydrate with G.C. mixture and then removing the non-aqueous solution by means of a separatory funnel: this was analyzed by the methods previously reported.[3, 5] The solution for the diffusion cell was then prepared by mixing weighed quantities

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Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, 1928–29, **12,** 543.

of two G.C. solutions so that the resultant solution contained two G.C. salts in equal concentration, about 0.3 M of each. This mixture was placed inside the cell and 25 cc. of G.C. mixture were then placed outside the cell and diffusion permitted long enough to produce a quantity sufficient for analysis.

The amounts diffusing for the pairs of alkalies were determined by shaking the G.C. solution with aqueous 0.1 M HCl and then determining the potassium or sodium content of the HCl extract. In all experiments with sodium present it was determined gravimetrically either by the sodium magnesium uranyl acetate method[8] or by the sodium zinc uranyl acetate method,[9] the latter being used when the quantity of sodium available was less than 8 mg. The other element present was determined by difference.

For the four pairs of alkali G.C. salts, each pair diffusing together in G.C. mixture containing them at equal concentration, the experiments showed the rate of diffusion to be the same within 12 per cent.

In the case of NH_4G the escape of NH_3 into the air was noticeable and in consequence no trustworthy values could be obtained. It seems fairly safe to assume that the diffusion constant of NH_4G does not differ much from those of K*G* and Na*G*.

No great accuracy can be claimed for these results, but they show that the diffusion constants are so similar that the differences between them may be neglected for our present purpose. But it is of interest to note that in general the heavier molecule appeared to diffuse faster which may be due to greater solvation on the part of the lighter molecule.

It is clear that such differences are not great enough to account for the large differences observed in the penetration of these substances in the model (Table I, p. 233). Let us now inquire how far these differences in penetration can be explained by the partition coefficients.

Since it is not possible to determine the partition coefficient S_{K_o}, *i.e.* (the concentration of undissociated K*G* in B_o) $\div$ (the concentration of potassium in A), we must content ourselves with determining[5] S_{KG_o}; *i.e.*, (the concentration of potassium in B_o) $\div$ (the concentration of potassium in A). There is probably little difference between these values since K*G* is a very weak electrolyte in the G.C. mixture.[5]

The partition coefficients are shown in Fig. 1. It will be noted that at lower concentrations the graphs approximate straight lines. This would be expected for reasons given in former papers.[2,4] At higher concentrations some of the curves bend over and may be

[8] Caley, E. R., and Foulk, C. W., *J. Am. Chem. Soc.*, 1929, **51**, 1664.

[9] Barber, H. H., and Kolthoff, I. M., *J. Am. Chem. Soc.*, 1928, **50**, 1625.

approaching a partition coefficient of unity. Determinations of K*G*, Na*G*, and Li*G* showed that the highest concentrations shown in Fig.

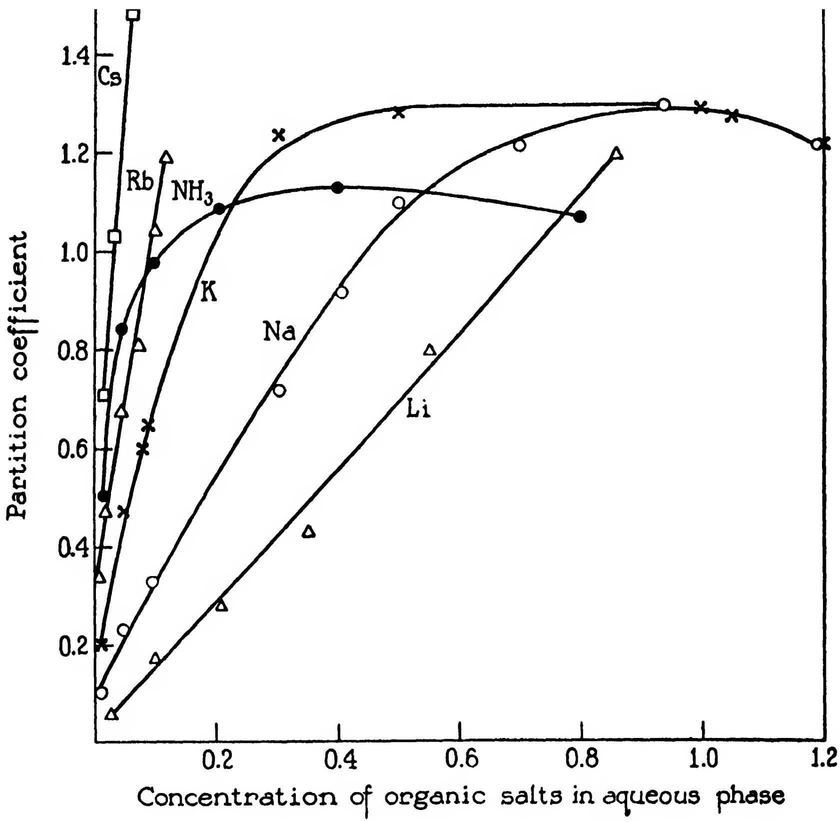

FIG. 1. Graphs showing values of partition coefficient $\frac{MG \text{ in non-aqueous phase}}{G^- \text{ in aqueous phase}}$, where M is an alkali cation: G^- in the aqueous phase is regarded as equal to M in the aqueous phase. Up to about 0.1 M the graphs are approximately straight lines. This would be expected on theoretical grounds. The curve is drawn free-hand to give an approximate fit to the observed values.

1 were not far from the plait points where the two phases fuse and the partition coefficients become unity.

Let us now consider the partition coefficients observed when a pair of salts (such as K*G* and Na*G*) are present simultaneously. In this case

it is not sufficient to take the values represented in Fig. 1 which shows, for example, that at 0.02 M *we have* $S_{KG_o} \div S_{NaG_o} = 0.25 \div 0.125 = 2$. The value of S_{KG_o} shown in the figure was obtained from a solution containing 0.02 M K*G* and that of S_{NaG_o} from a solution containing 0.02 M Na*G*. But we must now use[10] the values of S_{KG_o} and S_{NaG_o} in a solution containing 0.02 M K*G* + 0.02 M Na*G*. We then get $S_{KG_o} \div S_{NaG_o} = 0.35 \div 1.65 = 2.12$. The values of S_{KG_o} and S_{NaG_o} are higher than those shown in Fig. 1 because the concentration of guaiacol ion (G_o) and of (OH_o) is higher, which, as shown in previous papers, increases the value of the partition coefficient. This follows from the fact that K'_o is proportional to (K_o)(G_o) and to (K_o)(OH_o) when we take activities.[5] (We can calculate on this basis with sufficient accuracy for our purpose by using concentrations: this is demonstrated by making a series of determinations.)

The following experiments were made to ascertain the relation between partition coefficients and rates of entrance. The analytical procedure was similar to that for the diffusion experiments (p. 228). In all cases *C* contained distilled water at the start and CO_2 was bubbled continuously throughout the experiment.

(*a*) *Potassium and Lithium.*—The outer aqueous phase *A* contained 0.02 M K*G* + 0.02 M Li*G*. After 8 days the ratio K ÷ Li in *C* was 0.178 M ÷ 0.058 M = 3.1 (Experiment 108 *a*). A repetition gave in 8 days 0.250 M ÷ 0.088 M = 2.84 (Experiment 108 *b*). A similar experiment gave 0.0814 M ÷ 0.0261 M = 3.12 after 7 days (Experiment 108 *c*): the liquid in *C* was then removed and fresh distilled water (with CO_2 bubbling through it) was substituted. After 9 days the ratio in *C* was 0.0852 M ÷ 0.0229 M = 3.72 (Experiment 108 *d*). The average ratio was 3.09. Since the highest concentration reached in *C* was K*G* = 0.250 and Li*G* = 0.088 the system was far from the steady state.

A determination of the partition coefficients with 0.02 M K*G* + 0.02 M Li*G* in the aqueous phase gave $S_{KG_o} \div S_{LiG_o} = 0.414 \div 0.08 = 5.2$.

In the experiments here described Model II[3] was used for all the experiments in which Solution *A* contained the two alkalies at 0.02 M each. A constant flow was

[10] These determinations were made as described on p. 228. The resulting solution of chlorides was analyzed for potassium and sodium as already described (*cf.* footnote 3).

maintained in A: B contained 275 cc. of G.C. mixture and C 20 cc. of distilled water at the start, and CO_2 was bubbled continuously throughout the experiment. The temperature varied between 20 and 25°C. The other experiments listed in Table I, with the solution in A containing potassium and sodium at 0.05 M each and 0.1 M each, have been previously described.[3,5]

(*b*) *Sodium and Ammonium.*—The outer phase A[11] contained 0.02 M NaG + 0.02 M NH_4G. After 13 days the ratio in C was $NH_4 \div Na =$ 0.1925 M ÷ 0.0375 M = 5.1 (Experiment 106 *a*). A repetition gave after 8 days the ratio 0.1572 M ÷ 0.0238 M = 6.6 (Experiment 106 *b*): the solution in C was then replaced by distilled water in which CO_2 was bubbling and after 9 days we found 0.193 ÷ 0.0371 = 5.2 (Experiment 106 *c*). Fresh distilled water + CO_2 was placed in C and after 8 days we found 0.192 M ÷ 0.037 M = 5.2 (Experiment 106 *d*). Fresh distilled water plus CO_2 was again placed in C and after 7 days we found 0.1731 M ÷ 0.0289 M = 6.0. The average ratio was 5.6.

A determination of the partition coefficient with 0.02 M NaG + 0.02 M NH_4G in the aqueous phase gave $S_{NH_4G_o} \div S_{NaG_o} = 0.885 = 0.15 = 5.9$.

(*c*) *Rubidium and Sodium.*—The outer phase[11] A contained 0.02 M RbG + 0.02 M NaG. After 4 days the ratio in C was Rb ÷ Na = 0.0590 M ÷ 0.0252 M = 2.34 (Experiment 113 *a*). Fresh distilled water plus CO_2 was then placed in the C compartment. After 7 days the ratio in C was Rb ÷ Na = 0.1094 M ÷ 0.0372 M = 2.94 (Experiment 113 *b*).

A determination of the partition coefficient with 0.02 M RbG ÷ 0.02 M NaG in the aqueous phase gave $S_{RbG_o} \div S_{NaG_o} = 0.63 \div 0.181 = 3.5$.

(*d*) *Cesium and Sodium.*—The outer phase[11] A contained 0.02 M CsG + 0.02 M NaG. After 4 days the ratio in C was Cs ÷ Na = 0.0548 M ÷ 0.0115 M = 4.8 (Experiment 114 *a*). The solution in C was replaced by 20.0 cc. distilled water in which CO_2 was bubbling and after 9 days we found Cs ÷ Na = 0.0958 M ÷ 0.0228 M = 4.2.

A determination of the partition coefficients with 0.02 M CsG + 0.02 M NaG in the aqueous phase gave $S_{CsG_o} \div S_{NaG_o} = 1.29 \div 0.135 = 9.6$.

[11] The conditions were the same as in Experiment 108.

(*e*) *Potassium and Sodium.*—The outer phase *A* contained 0.02 M KG + 0.02 M NaG. After 10 days the ratio K ÷ Na in *C* was 0.103 M ÷ 0.042 M = 2.45 (Experiment 109 *a*). A repetition gave in 8 days 0.097 M ÷ 0.041 M = 2.37 (Experiment 109 *b*). A third experiment gave 0.107 M ÷ 0.049 M = 2.28 after 11 days (Experiment 109 *c*). The average ratio was 2.37.

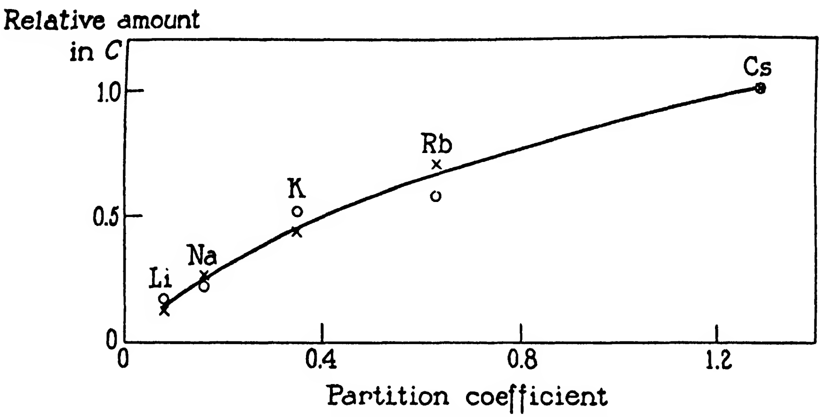

FIG. 2. Graph showing that the salt which predominates in *C* is the one with the higher partition coefficient, which is defined as $\frac{MG \text{ in non-aqueous phase}}{G^{-} \text{ in aqueous phase}}$, where *M* is an alkali cation: G^{-} in the aqueous phase is regarded as equal to *M* in the aqueous phase. The partition coefficients are plotted as abscissae (see Table II, p. 234). The relative amount in *C* is plotted as ordinates, that of cesium being taken as unity (see Table II). The curve is drawn free-hand to give an approximate fit to the observed values. Observed values, o; calculated values, ×.

The partition coefficients for 0.02 M KG + 0.02 M NaG interpolated from determinations at several concentrations for these salts (when present in equal concentration) are $S_{KG_o} \div S_{NaG_o} = 0.35 \div 0.165 = 2.1$.

The results are summarized in Tables I and II. Fig. 2 shows[12] that as the partition coefficient increases the relative amount found in *C* also increases but the curve flattens out rather rapidly. This would

[12] For reasons which will be discussed presently ammonium is omitted from this figure.

TABLE I

Summary of Penetration Experiments with Alkali Salts

Experiment	Time	Ratio found in C	Solution in A	Ratio of partition coefficients
		K + Na		
	days			
58	9	1.4	0.1 M KG + 0.1 M NaG	$S_{KG_o} \div S_{NaG_o} = 2.3$
63	12	1.5		
		Av. = 1.45		
66	33 Steady state	1.6	0.05 M KG + 0.05 M NaG	$S_{KG_o} \div S_{NaG_o} = 2.14$
80	4 Steady state	2.7		
81	4 Steady state	2.7		
111	50 Steady state	2.0		
		Av. = 2.25		
109 *a*	10	2.45	0.02 M KG + 0.02 M NaG	$S_{KG_o} \div S_{NaG_o} = 2.1$
b	8	2.37		
c	11	2.28		
		Av. = 2.37		
		K + Li		
108 *a*	8	3.1	0.02 M KG + 0.02 M LiG	$S_{KG_o} \div S_{LiG_o} = 5.2$
b	8	2.84		
c	7	3.12		
d	9	3.72		
		Av. = 3.09		
		NH_4 + Na		
106 *a*	13	5.1	0.02 M NH_4G + 0.02 M NaG	$S_{NH_4G_o} \div S_{NaG_o} = 5.9$
b	8	6.6		
c	9	5.2		
d	8	5.2		
e	7	6.0		
		Av. = 5.62		
		Rb + Na		
113 *a*	4	2.34	0.02 M RbG + 0.02 M NaG	$S_{RbG_o} \div S_{NaG_o} = 3.5$
b	6	2.94		
		Av. = 2.64		
		Cs + Na		
114 *a*	4	4.8	0.02 M CsG + 0.02 M NaG	$S_{CsG_o} \div S_{NaG_o} = 9.6$
b	9	4.2		
		Av. = 4.50		

be expected on the basis of the calculation given in the previous paper[13] where a method is presented for calculating $K_i \div Na_i$ after the first increment of time. Employing this we obtain the values given in Column 3 of Table II.

The agreement with the observed values is surprisingly good when it is remembered that (in addition to the difficulties mentioned in the previous paper[13]) the determinations were not made at the beginning but at later stages and even in the steady state.

TABLE II

Relative Amounts Found in C, That of Cesium being Taken As Unity (in Determining These the Averages for 0.02 M in Table I Were Employed)

	Relative amount in *C*		Partition coefficient
	Observed	Calculated	
Cs	1.00	1.00	1.29
Rb	0.58	0.71	0.63
K	0.52	0.44	0.35*
Na	0.22	0.26	0.165*
Li†	0.17	0.13	0.08
NH_4	1.25		0.88

* The partition coefficient of Na*G* depends on the substance with which it is paired: thus with K*G* it is 0.165, with Rb*G* 0.181, with Cs*G* 0.135, and with NH_4G 0.152: the partition coefficient of K*G* is 0.414 with Li*G*, and 0.35 with Na*G*. In constructing Fig. 2 it was necessary to take single values and those given in Table II were selected as the best established since more determinations were made. The differences in the values for S_{KG_o} and S_{NaG_o} depending on the alkali accompanying it may be due to a number of factors, including variations in temperature and in the composition of the G.C. mixture.

† The relative amount (both calculated and observed) of lithium was related to that of cesium by relating cesium to sodium, sodium to potassium, and, finally, potassium to lithium.

Furthermore in these experiments CO_2 was bubbling in *C* and water was entering: neither of these factors was taken into account in the calculation.

We see that both calculation and observation indicate that the rate

[13] Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933–34, **17**, 449.

of penetration is not directly proportional to the partition coefficient, but increases somewhat less rapidly. This may also be the case with living cells.

DISCUSSION

The order of partition coefficients is

$$Cs > NH_4 > Rb > K > Na > Li.$$

That of rates of penetration is

$$NH_4 > Cs > Rb > K > Na > Li.$$

The fact that ammonium appears to penetrate rapidly is quite in accord with biological experience for we find that in general its penetration into living cells is relatively rapid. The reason usually given is that in addition to penetrating as ammonium (either free or combined) it can penetrate as NH_3. This would also apply to the models. If, for example, *B* contained undissociated NH_4G, NH_4OH, and NH_3, the last would have the highest diffusion constant on account of its small molecular weight. In addition to this it is quite possible that the partition coefficient is really higher than the analysis shows owing to loss of NH_3 during the determination. We may therefore regard ammonium as not strictly comparable to the other salts.

Considering only cesium, rubidium, potassium, sodium, and lithium we see that the order of penetration corresponds to that of their partition coefficients and to that of the ionic mobilities in water. Hence it would seem that similar causes determine the order of ionic mobilities in water and the order of partition coefficients. This will be discussed in forthcoming papers by physical chemists: since their work indicates that these salts are weak electrolytes in *B* we may conclude that ionic transport in *B* plays a subordinate rôle and that the partition coefficients are chiefly responsible for the order of penetration.

The predominating effect of the partition coefficient is also shown in experiments with KCl. Here the diffusion coefficient is presumably greater than for K*G* but in spite of this the rate of penetration is extremely small, corresponding to the very small partition coefficient.

SUMMARY

In some living cells the order of penetration of certain cations corresponds to that of their mobilities in water. This has led to the

idea that electrolytes pass chiefly as ions through the protoplasmic surface in which the order of ionic mobilities is supposed to correspond to that found in water.

If this correspondence could be demonstrated it would not prove that electrolytes pass chiefly as ions through the protoplasmic surface for such a correspondence could exist if the movement were mostly in molecular form.

This is clearly shown in the models here described. In these the protoplasmic surface is represented by a non-aqueous layer interposed between two aqueous phases, one representing the external solution, the other the cell sap.

The order of penetration through the non-aqueous layer is

$$\text{Cs} > \text{Rb} > \text{K} > \text{Na} > \text{Li}.$$

This will be recognized as the order of ionic mobilities in water. Nevertheless the movement is mostly in molecular form in the non-aqueous layer (which is used in the model to represent the protoplasmic surface) since the salts are very weak electrolytes in this layer.

The chief reason for this order of penetration lies in the fact that the partition coefficients exhibit the same order, that of cesium being greatest and that of lithium smallest.

The partition coefficients largely control the rate of entrance since they determine the concentration gradient in the non-aqueous layer which in turn controls the process of penetration. The relative molecular mobilities (diffusion constants) in the non-aqueous layer do not differ greatly. The ionic mobilities are not known (except for K^+ and Na^+) but they are of negligible importance, since the movement in the non-aqueous layer is largely in molecular form. They may follow the same order as in water, in accordance with Walden's rule.

Ammonium appears to enter faster than its partition coefficient would lead us to expect, which may be due to rapid penetration of NH_3. This recalls the apparent rapid penetration of ammonium in living cells which has also been explained as due to the rapid penetration of NH_3.

Both observation and calculation indicate that the rate of penetration is not directly proportional to the partition coefficient but increases somewhat less rapidly.

Many of these considerations doubtless apply to living cells.

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THE THEORY OF DIFFUSION IN CELL MODELS

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I

INTRODUCTION

Osterhout and Stanley[1] have recently described some interesting experiments on diffusion between two aqueous phases separated by a non-aqueous phase. These experiments were modeled after living cells and duplicated the power of some cells to concentrate preferentially certain chemical substances. Furthermore, the volume increase shown by one of the aqueous phases in the model is somewhat analogous to the growth of living cells. In a model in which water and a salt are the only substances whose diffusion need be considered Osterhout[2] has shown that the results are in qualitative accord with the kinetics which characterize two consecutive monomolecular reactions. It seems to the author, however, that the mechanism more nearly corresponds to that of two simultaneous and mutually dependent processes. Consequently it is the purpose of this paper to formulate and solve the simultaneous differential equations which probably describe the model. These equations will be derived starting with the simple but fundamental laws which describe diffusion processes in general.

Jacobs[3] has recently formulated the simultaneous differential equations which describe the relation between cell volume and penetration of a solute. These equations, though similar to the ones which are derived in this paper, are somewhat more difficult to solve and the desired time curves are not obtained by direct integration.

[1] Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931–32, **15**, 667

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1932–33, **16**, 529.

[3] Jacobs, M. H., *J. Cellular and Comp. Physiol.*, 1933, **3**, 29.

II

Description of the Experiment

Osterhout[2] has given a detailed description of the experiment which is to be treated theoretically in this paper. He has also included an excellent discussion of the physical processes which are involved and the justification for certain assumptions which it will be necessary to make in the development of the theory. Therefore only a brief discussion of the experiment will be given in the following paragraphs.

The aqueous layer *A* (Fig. 1) consists of water saturated with a weak acid, H*G*, and contains the potassium salt of this acid, K*G*, at a concentration of 0.05 normal. The solution in *A* is continuously replaced during the experiment so as to maintain a constant concentration of K*G* in this region. The non-aqueous layer *B* consists of

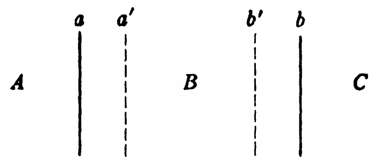

Fig. 1

the weak acid H*G* which is but slightly miscible with water and which is initially saturated with the latter. This phase separates *A* from a second aqueous layer *C* which is saturated with H*G* and through which CO_2 is continuously bubbled at atmospheric pressure. All three phases are stirred so that the diffusion which occurs takes place in the layers at the phase boundaries *a* and *b* which are not affected by the stirring. As Osterhout has pointed out the high viscosity of the non-aqueous medium makes it probable that the unstirred layers are much thicker in this phase than in the outer aqueous layers. It will therefore be assumed that the only gradients of composition which occur are in the layers *aa'* and *bb'* (Fig. 1). The mobilities of the diffusing constituents are much lower in the non-aqueous phase[4] than in the aqueous phases, an additional fact which makes it probable that the constants for the diffusion processes are determined largely by the characteristics of phase *B*.

[4] Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933–34, **17**, in press, (Kinetics. VI).

The two substances whose simultaneous diffusion through B is significant are KG and H_2O. The force which causes the diffusion of KG, for example, from A to C is the gradient of chemical potential for this substance which exists between A and C due to the difference in the concentration of this substance in the two layers. The chemical potential μ_i, of any given molecular species i may be defined by means of the equations[5]

$$\mu_i = \mu_i^0 + RT \ln a_i \tag{2.1}$$

$$= \mu_i^0 + RT \ln \gamma_i N_i \tag{2.2}$$

In these equations μ_i^0 is the chemical potential in some arbitrarily chosen standard state, R the gas constant, T the absolute temperature, a_i the activity, γ_i the activity coefficient, and N_i the concentration of the i-th constituent. The concentration scale best adapted for the treatment of the present problem will be considered in the following section.

The KG diffuses from A in which the concentration, and hence the chemical potential, of this substance is maintained at a constant value, through B into C where its concentration is initially zero and where the following reaction occurs.

$$\mathrm{K}G + \mathrm{H_2CO_3} = \mathrm{KHCO_3} + \mathrm{H}G \tag{2.3}$$

The mass action expression for this reaction is

$$a_{\mathrm{H}G} \cdot a_{\mathrm{KHCO_3}} = a_{\mathrm{K}G} \cdot a_{\mathrm{H_2CO_3}} \cdot \text{constant} \tag{2.4}$$

Since the partial pressure of CO_2 is constant and since the activity of HG in C is maintained at a substantially constant value by contact with the non-aqueous phase in which HG predominates, equation (2.4) may be simplified to

$$a_{\mathrm{KHCO_3}} = K \cdot a_{\mathrm{K}G}$$

K in this expression being a constant.

[5] Lewis, G. N., and Randall, M., Thermodynamics, New York, McGraw-Hill Book Co., Inc., 1923, 254. See also Gibbs, J. W., Collected works, Longmans, Green and Co., New York, 1928, 1, 92.

If the salt activities in this expression are replaced by the products of the ion constituent activities according to the convention of Lewis and Randall (p. 326 of footnote 5)

$$a_{K^+} \cdot a_{HCO_3^-} = K\, a_{K^+} \cdot a_{G^-}$$

or

$$a_{HCO_3^-} = K\, a_{G^-} \tag{2.5}$$

Since the activity coefficients which may be inserted into equation (2.5) in order to convert the activities to concentrations always occur as a ratio, it follows from the principle of the ionic strength that a very close approximation to (2.5) will be obtained by placing

$$N_{KHCO_3} = K \cdot N_{KG} \tag{2.6}$$

Since H_2CO_3 is a much stronger acid than HG the constant of equation (2.6) has a value much greater than unity and most of the KG which diffuses into C is converted into $KHCO_3$.

The back diffusion of $KHCO_3$ from C to A may be neglected due to the very slight solubility of this substance in the non-aqueous phase. CO_2 appears to be able to diffuse from C to A. This does not occur to any appreciable extent in the model, however, and may therefore be neglected. It is possible that some CO_2 does diffuse across the phase boundary b but if the reaction represented by equation (2.3) occurs in phase B the $KHCO_3$ thus formed would be immediately extracted by the aqueous layer C, thus compensating for the diffusion of CO_2.

Whereas the chemical potential gradient causing the diffusion of KG from A to C will depend upon the value of N_{KG} in C the potential or osmotic pressure of the water in this phase will depend upon the total concentration of solute in C. Since the chief solute in C is $KHCO_3$ the diffusion of KG from A to C with subsequent conversion into $KHCO_3$ will eventually lower the activity of the water in C to a value below that in A. The effect of this upon the diffusion of water will now be considered.

At the beginning of the experiment, when the concentration of solute in C is lower than in A, water will diffuse from C into A. This

initial movement of H_2O from C to A will continue until the diffusion of KG in the reverse direction, followed by its conversion into $KHCO_3$ in C, builds up the solute concentration in this phase to such a value that the activities of the water in A and C are equal. The activity of KG in C, however, which corresponds to this concentration of $KHCO_3$ is still much lower than the activity of KG in A so that KG continues to enter C and be converted into $KHCO_3$. This lowers the activity of H_2O in C below its value in A and the water consequently reverses its direction of flow and henceforth moves from A to C. The time at which this reversal occurs corresponds to a minimum in the water content of the phase C and is treated further in Section IX.

The simultaneous movement of KG and H_2O from A to C then continues indefinitely and approaches a condition in which KG and H_2O enter C in essentially the same ratio as that of $KHCO_3$ and H_2O already present, so that on conversion of the entering KG into $KHCO_3$ the concentration of this latter substance in C remains unaltered. This condition is called the steady state and is an important feature of the experiment.

Since it is impossible to fix rigidly the phase B in the model, the hydrodynamic pressure developed by the increase in the volume of C is exactly compensated by a shift in the position of the intermediate phase and must consequently be neglected in the present theory.

III

The Concentration Scale

As will be shown later, it is necessary to consider the partition of the diffusing constituents between the various phases and since one of these, water, is that which, in the outer aqueous layers, would normally be termed the solvent a concentration scale must be selected which is symmetrical with respect to all constituents. The mole fraction satisfies this requirement and will therefore be adopted. As indicated in equation (2.2) N_i represents the mole fraction of the i-th constituent while the actual number of moles of i present in any given region will be denoted by n_i. The mole fraction is defined by the expression

$$N_i = \frac{n_i}{n_0 + n_1 + n_2 + \cdots} = \frac{n_i}{\Sigma n_i} \tag{3.1}$$

in which the summation is to be taken over all species present. The *mole fraction is related to the volume concentration* (c_i moles per milliliter of solution) by the equation

$$c_i = \frac{n_i}{\Sigma n_i \overline{V}_i}$$

in which $\overline{V}_i$ is the partial molal volume of the i-th constituent in milliliters.

If one constituent of a phase is greatly predominant it may be represented by the subscript 0 and no serious error is involved in replacing the mole fraction of another constituent by its mole ratio, $\frac{n_i}{n_0}$,

In this case

$$\left.\begin{aligned} &N_i \simeq \frac{n_i}{n_0};\ N_0 \simeq 1 - \frac{\Sigma n_i}{n_0} \qquad (3.2)\\ &c_i \simeq \frac{n_i}{n_0 V_0} \simeq \frac{N_i}{V_0} \qquad (3.3)\end{aligned}\right\} i \neq 0$$

in which V_0 is the molal volume of the "solvent."

IV

The Diffusion Equation

In the derivation of the equations which describe molecular diffusion processes it is customary to assume that the velocity of migration, v_i, of the i-th constituent is proportional to the chemical potential gradient, $\frac{d\mu_i}{dx}$, of that substance[6]

$$v_i = -u_i \frac{d\mu_i}{dx} \tag{4.1}$$

u_i being the factor of proportionality or mobility. The derivatives of μ_i with respect to the other space coordinates may be neglected if diffusion takes place in one direction only as in the present experiment. Equation (4.1) may be multiplied by N_i and rearranged to

[6] Onsager, L., and Fuoss, R. M., *J. Phys. Chem.*, 1932, **36**, 2689 (see page 2759).

$$v_i N_i = -u_i N_i \frac{d\mu_i}{dN_i} \frac{dN_i}{dx} \tag{4.2}$$

Since this equation is to be applied only to the substances diffusing through B and since these are present in this phase at relatively low volume concentrations the approximation represented by equation (3.3) may be introduced into equation (4.2) to give

$$v_i c_i = -\frac{u_i N_i}{V_0} \frac{d\mu_i}{dN_i} \frac{dN_i}{dx}$$

It is evident, however, that the product $v_i c_i$ is the velocity with which a given concentration moves and hence is the flux of material per unit area and unit time, $\frac{\partial n_i}{\partial t}$, through a plane perpendicular to the direction of migration. If A is the total cross-section of the diffusion layer the diffusion equation becomes

$$\frac{\partial n_i}{\partial t} = -\frac{A}{V_0} \left(u_i N_i \frac{d\mu_i}{dN_i} \right) \frac{\partial N_i}{\partial x} \tag{4.3}$$

Equation (4.3) will be recognized as Fick's law if

$$D_i = u_i N_i \frac{d\mu_i}{dN_i}$$

D_i being the diffusion coefficient. By means of equation (2.2) this may be rearranged to

$$D_i = RT\, u_i \left(1 + \frac{d \ln \gamma_i}{d \ln N_i} \right) \tag{4.4}$$

Since $\frac{d \ln \gamma_i}{d \ln N_i}$ is generally a function of N_i it is evident that the diffusion coefficient will also vary with the concentration. However, it has already been assumed that diffusion occurs only in the non-aqueous phase, in which, due to the low ionizing power of the solvent, electrolytes behave more nearly as perfect solutes than in an aqueous phase. For a perfect solute the differential coefficient in equation (4.4) is zero and in the present instance may at least be assumed constant.

Moreover, it simplifies the mathematical treatment if the very probable assumption is made that at any given time the thickness of

the combined diffusion layers (aa' and bb' of Fig. 1) has a ... ıffi-ciently small to warrant the consideration of the concentration gradient as constant and equal to $\frac{\Delta N_i}{\Delta x}$. Since the value of Δx is nearly independent of the time, equation (4.3) may be rewritten

$$\frac{dn_i}{dt} = -\frac{AD_i}{V_0 \cdot \Delta x} \Delta N_i \tag{4.5}$$

V

Equilibria at the Phase Boundaries

Equation (4.5) is the general differential equation which will be adapted to the experiment considered in this paper. Before this adaptation can be made, however, the mechanism of the transfer of material across the phase boundaries must be considered. If, as is usually assumed, *equilibrium for a given constituent is attained practically instantaneously at a phase boundary, it is correct to say that the chemical potential for that substance is equal in the two phases at the boundary and hence has no discontinuity at that place.*

According to equation (4.1) the velocity of a constituent is proportional to the gradient of chemical potential, the latter being continuous at a phase boundary as indicated above, but the flux of a constituent is determined not only by its velocity but by the product of this into the concentration which is usually discontinuous at a phase boundary. It thus appears that the driving force which causes diffusion across the non-aqueous layer will be determined largely by the composition of the two aqueous phases, whereas the flux of material will depend in part upon the concentration of the diffusing substances in the non-aqueous layer. Thus, other conditions being the same, a given difference of composition between A and C will cause a greater flux of some constituent through B the more soluble that constituent is in the non-aqueous layer. As Osterhout[2] has emphasized, partition coefficients will therefore play an important rôle in the present theory and may be defined for the phase boundaries a and b by the relations

$$S_i^a = N_i^{Ba}/N_i^A \tag{5.1}$$

$$S_i^b = N_i^{Bb}/N_i^C \tag{5.2}$$

In these expressions the superscript denotes the region to which the concentration or partition coefficient refers. Thus N_i^{Ba} is the concentration of the i-th constituent in phase B at the plane a. These are stoichiometric partition coefficients and are functions of the concentration in general.

Since the aqueous layer A is continuously replaced, N^A_{KG}, $N^A_{H_2O}$ and the corresponding partition coefficients, S^a_{KG} and $S^a_{H_2O}$, are constants, the values of the latter being 1.7[4] and 0.417[7] respectively. At the phase boundary b, however, the concentrations, and hence the partition coefficients, vary with the time and this variation on the part of the partition coefficients must now be discussed in some detail.

In the absence of data on the effect of salts upon the distribution of the non-electrolyte, water, the coefficient $S^b_{H_2O}$ may be considered constant—and equal to 0.417, the value obtained from the mutual solubility of the two liquids, HG and H_2O, in the absence of salts—if the effect of the salt ionization upon the activity of water in the aqueous phase is considered. This may be done by introducing the osmotic coefficient, i, into the expressions for $N^A_{H_2O}$ and $N^C_{H_2O}$, as follows:

$$N^C_{H_2O} \simeq 1 - N^C_{HG} - N^C_{CO_2} - i^C N^C_{KHCO_3} \tag{5.3}$$

$$N^A_{H_2O} \simeq 1 - N^A_{HG} - i^A N^A_{KG} \tag{5.4}$$

The osmotic coefficient is also a function of the concentration but no serious error will be made if an average but constant value is assigned to it for the particular experiment under consideration. Thus a value of 1.9 may be assigned to i^A, corresponding to the constant value of $N^A_{KG} = 0.00090$, and $i^C \simeq 1.7$ which corresponds to the average value $N^C_{KHCO_3} = 0.006$ (see Table I). The values of N^A_{HG} ($= N^C_{HG}$) and $N^C_{CO_2}$ are 0.0027[1] and 0.00062,[8] respectively.

[7] Unpublished measurements made in this laboratory. The solubility of water in the non-aqueous material is about 9.73 per cent. Thus 100 gm. of the non-aqueous phase contain 9.73 gm. of H_2O or 0.54 mole, and 90.3 gm. of HG or 0.756 mole (see Footnote 12). $N^{Ba}_{H_2O}$ is therefore $\dfrac{0.54}{0.54 + 0.756}$ or 0.417 and since $N^A_{H_2O}$ is essentially unity, $S^a_{H_2O} = 0.417$.

[8] International Critical Tables, New York, McGraw-Hill Book Co., Inc., 1928, 3, 260.

Osterhout, Kamerling, and Stanley[4] have found that the distribution of KG between HG and H_2O is quite sensitive to the concentration when this is low but becomes nearly independent of the latter as saturation is approached. The exact functional relationship has not been established, however, and in the present theory a constant value of 1.85 will be assigned to S^b_{KG}. The use of a constant value for S^b_{KG} in this manner is clearly unsatisfactory and probably represents a rather poor approximation, but the mathematical complexity which results from the assumption of even a linear variation of the partition coefficient with concentration is rather serious. Moreover the common ion effect of the potassium ion from the $KHCO_3$ in C upon the partition of KG introduces an additional complication. The value which has been assigned to S^b_{KG} was obtained in the following manner.

According to the conventions of Lewis and Randall[5] the activity of KG in the phase C is given by the relation

$$a^C_{KG} = a^C_{K^+} \cdot a^C_{G^-} = (\gamma^C_\pm)^2 \, N^C_{K^+} \cdot N^C_{G^-}$$

in which $\gamma^C_\pm$ is the mean ion activity coefficient and will depend upon the total ionic strength of phase C. The distribution measurements of Osterhout, Kamerling, and Stanley were made with solutions of pure KG and in this case,

$$\overline{N}^C_{K^+} = \overline{N}^C_{G^-} = \overline{N}^C_{KG}$$

and

$$a^C_{KG} = (\overline{\gamma}^C_\pm)^2 \, (\overline{N}^C_{KG})^2 \tag{5.5}$$

the bar over the symbol indicating that KG is the only salt present. In the experiment being considered in this paper, however, $KHCO_3$ is also present in C at an average concentration of $N^C_{KHCO_3} = 0.006$, so that

$$N^C_{K^+} = N^C_{KHCO_3} + N^C_{KG}$$

$$N^C_{G^-} = N^C_{KG}$$

and

$$a^C_{KG} = (\gamma^C_\pm)^2 \, N^C_{KG} \, (N^C_{KHCO_3} + N^C_{KG}) \tag{5.6}$$

Elimination of a^C_{KG} between equations (5.5) and (5.6) gives

$$\overline{N}^C_{KG} = \frac{\gamma^C_{\pm}}{\overline{\gamma}^C_{\pm}} \sqrt{N^C_{KG}\,(N^C_{KHCO_3} + N^C_{KG})} \tag{5.7}$$

or with reference to equation (2.6)

$$\overline{N}^C_{KG} = \frac{\gamma^C_{\pm}}{\overline{\gamma}^C_{\pm}} N^C_{KHCO_3} \sqrt{\frac{1}{K} + \frac{1}{K^2}}$$

By means of equations (6.8) and (8.4) which follow, K may be eliminated to give

$$\overline{N}^C_{KG} = \frac{\gamma^C_{\pm}}{\overline{\gamma}^C_{\pm}} N^C_{KHCO_3} \sqrt{\frac{0.0876}{S^b_{KG}} + \frac{0.00768}{(S^b_{KG})^2}}$$

The value of S^b_{KG} corresponding to this value of $\overline{N}^C_{KG}$, as obtained from a plot of the partition measurements mentioned above, is the quantity desired. Since S^b_{KG} must be known, however, before $\overline{N}^C_{KG}$ can be evaluated and since $\overline{\gamma}^C_{\pm}$ also depends upon N^C_{KG}, a series of approximations will be necessary. For $N^C_{KHCO_3} = 0.006$, $\gamma^C_{\pm} \sim 0.59$.[9] The computed value $\overline{N}^C_{KG}$ is 0.00106, so that $S^b_{KG} = 1.85$ and $\overline{\gamma}^C_{\pm} \sim 0.73$ if the activity coefficients of KG are similar to those for $KHCO_3$ and if the small amount of HG present in the aqueous phase C may be ignored. In the steady state $N^C_{KHCO_3} = 0.012$ and a similar calculation gives the value 2.5 for S^b_{KG}. The difference between 1.85 and 2.5 affords an estimate of the error which is involved in the assignment of a constant value to S^b_{KG}.

It has been necessary to develop the foregoing theory in order to utilize the results of ordinary partition measurements in obtaining the distribution equilibria in the presence of an added salt with a common ion. It should be apparent that the theory is also applicable when a salt with no ion in common is added to the aqueous phase. The effect upon the partition equilibria is then contained entirely in the activity coefficient ratio of equation (5.7). However, it should be emphasized that the added salt cannot be appreciably soluble in the non-aqueous phase if the foregoing equations are to be applicable.

[9] Guntelberg, E., and Schiödt, E., *Z. phys. Chem.*, 1928, **135**, 393.

VI

Specialization of the Diffusion Equation

The flux of the two diffusing substances through the plane b, Fig. 1, will now be considered. The flux of KG through this plane is, according to equation (4.5),

$$\left(\frac{dn_{KG}}{dt}\right)_b = \frac{AD_{KG}}{V_{HG}\Delta x}\,(N_{KG}^{Ba} - N_{KG}^{Bb})$$

From equations (5.1) and (5.2), this may also be written

$$\left(\frac{dn_{KG}}{dt}\right)_b = \frac{AD_{KG}}{V_{HG}\Delta x}\,(S_{KG}^a\,N_{KG}^A - S_{KG}^b\,N_{KG}^C) \tag{6.1}$$

Due to the reaction (see equation (2.3)) which occurs in C the flux of KG through the plane b is essentially the rate of appearance of $KHCO_3$ in C

$$\left(\frac{dn_{KG}}{dt}\right)_b = \frac{dn_{KHCO_3}^C}{dt}$$

Elimination of N_{KG}^C between equations (6.1) and (2.6) gives

$$\frac{dn_{KHCO_3}^C}{dt} = \frac{AD_{KG}}{V_{HG}\Delta x}\left(S_{KG}^a\,N_{KG}^A - \frac{S_{KG}^b}{K}\,N_{KHCO_3}^C\right) \tag{6.2}$$

Similarly the rate of appearance of water in C is

$$\frac{dn_{H_2O}^C}{dt} = \frac{AD_{H_2O}}{V_{HG}\Delta x}\,(S_{H_2O}^a\,N_{H_2O}^A - S_{H_2O}^b\,N_{H_2O}^C) \tag{6.3}$$

Since the partition coefficients are to be considered constant, reference to equations (3.2), (5.3), and (5.4) shows that the number of dependent variables in equations (6.2) and (6.3) may be reduced to two as follows:

$$\frac{dn_{KHCO_3}^C}{dt} = \frac{AD_{KG}}{V_{HG}\Delta x}\left(S_{KG}^a\,N_{KG}^A - \frac{S_{KG}^b}{K}\,\frac{n_{KHCO_3}^C}{n_{H_2O}^C}\right)$$

$$\frac{dn_{H_2O}^C}{dt} = \frac{AD_{H_2O}}{V_{HG}\Delta x}\,S_{H_2O}\left[(1 - N_{HG}^A - i^A N_{KG}^A) - \left(1 - N_{HG}^C - N_{CO_2}^C - i^C\,\frac{n_{KHCO_3}^C}{n_{H_2O}^C}\right)\right]$$

$$= \frac{AD_{H_2O}}{V_{HG}\Delta x}\,S_{H_2O}\left(-\,i^A\,N_{KG}^A + N_{CO_2}^C + i^C\,\frac{n_{KHCO_3}^C}{n_{H_2O}^C}\right)$$

The notation may be simplified by placing

$$n^C_{KHCO_3} = n_1 \tag{6.4}$$

$$n^C_{H_2O} = n_0 \tag{6.5}$$

and by collecting the various constants as follows:

$$\frac{AD_{KG}}{V_{HG}\Delta x}\frac{S^b_{KG}}{K} = \alpha_1 \tag{6.6}$$

$$\frac{AD_{H_2O}}{V_{HG}\Delta x} S_{H_2O} \cdot i^C = \alpha_0 \tag{6.7}$$

$$N^A_{KG}\, K \cdot S^a_{KG}/S^b_{KG} = \beta_1 \tag{6.8}$$

$$N^C_{CO_2}/i^C - N^A_{KG}\, i^A/i^C = -\beta_0 \tag{6.9}$$

Then

$$\frac{dn_1}{dt} = \alpha_1\left(\beta_1 - \frac{n_1}{n_0}\right) \tag{6.10}$$

$$\frac{dn_0}{dt} = \alpha_0\left(-\beta_0 + \frac{n_1}{n_0}\right) \tag{6.11}$$

VII

Solution of the Differential Equations

Elimination of n_1/n_0 between equations (6.10) and (6.11) gives the expression

$$\frac{1}{\alpha_1}\frac{dn_1}{dt} + \frac{1}{\alpha_0}\frac{dn_0}{dt} = \beta_1 - \beta_0$$

which may be immediately integrated to give

$$\frac{n_1}{\alpha_1} + \frac{n_0}{\alpha_0} = (\beta_1 - \beta_0)t + \frac{I_0}{\alpha_0} \tag{7.1}$$

$\frac{I_0}{\alpha_0}$ is a constant of integration, I_0 being merely the number of moles of water initially present in phase C since the value of n_1 is zero at this time.

Translation of the time axis by means of the substitution

$$t = t' - \frac{I_0}{\alpha_0(\beta_1 - \beta_0)}$$

reduces equation (7.1) to

$$\frac{n_1}{\alpha_1} + \frac{n_0}{\alpha_0} = (\beta_1 - \beta_0)t' \tag{7.2}$$

Equation (7.2) tells us nothing about the individual dependence of n_1 and n_0 upon the time, but by solving this expression for n_1 and substituting this value into equation (6.11) an expression involving only one dependent variable is obtained. Thus,

$$n_1 = \alpha_1(\beta_1 - \beta_0)t' - \frac{\alpha_1}{\alpha_0} n_0$$

and

$$\frac{dn_0}{dt'} = -\alpha_0\beta_0 - \alpha_1 + \alpha_1\alpha_0(\beta_1 - \beta_0)\frac{t'}{n_0} \tag{7.3}$$

Equation (7.3) is homogeneous and the variables are rendered separable by the substitution

$$n_0 = v \cdot t' \tag{7.4}$$

so that it becomes

$$vdt' + t'dv = -bdt' - a\frac{dt'}{v}$$

if

$$-a = \alpha_1\alpha_0(\beta_1 - \beta_0) \tag{7.5}$$

$$b = \alpha_1 + \alpha_0\beta_0 \tag{7.6}$$

Rationalization and separation of the variables gives the expression

$$\frac{vdv}{a + bv + v^2} + \frac{dt'}{t'} = 0$$

The integral of this is

$$\ln(a + bv + v^2) - \frac{b}{\sqrt{-q}}\ln\frac{2v + b - \sqrt{-q}}{2v + b + \sqrt{-q}} + 2\ln I_1' t' = 0 \tag{7.7}$$

in which I_1' is a constant of integration and $q = 4a - b^2$.
Since

$$4(a + bv + v^2) = (2\,v + b - \sqrt{-q})\,(2\,v + b + \sqrt{-q})$$

equation (7.7) may be rearranged to

$$\left(1 - \frac{b}{\sqrt{-q}}\right)\log\,(2\,v + b - \sqrt{-q}) + \left(1 + \frac{b}{\sqrt{-q}}\right)\log\,(2\,v + b + \sqrt{-q}) + 2\log t' + I_1 = 0 \tag{7.8}$$

In equation (7.8) Briggsian logarithms have been substituted throughout for the natural logarithms and it is in this form that computations can be made most conveniently. This equation cannot be solved explicitly for v, and hence for n_0, but it is already explicit in t' and therefore may be readily employed for a calculation of the time curves as follows:

From equations (7.2) and (7.4),

$$N_1 \simeq \frac{n_1}{n_0} = \alpha_1(\beta_1 - \beta_0)\,\frac{t'}{n_0} - \frac{\alpha_1}{\alpha_0} = \alpha_1(\beta_1 - \beta_0)\,\frac{1}{v} - \frac{\alpha_1}{\alpha_0} \tag{7.9}$$

and a value of v may be computed which corresponds to a given concentration, N_1. This value of v is substituted in equation (7.8) and the corresponding value of t' computed. This value of t', together with the value of v which was taken, is then substituted in equation (7.4) and n_0 computed, etc.

Before these computations can actually be made, however, values must be assigned to the necessary constants. The constant I_0, as shown above, is the number of moles of water initially present in phase C and has the value 3.321. A value of 0.00064 for β_0 may be computed from equation (6.9) since all of the terms on the left hand side of this expression are known. After values have been assigned to the other necessary constants the integration constant I_1 may be evaluated by means of equation (7.8) from the conditions that at zero time $t' = \dfrac{I_0}{\alpha_0(\beta_1 - \beta_0)}$ and $v = \dfrac{I_0}{t'}$ (equation (7.4)). The value of I_1 which was used in the computations for the time curves is -2.202297. The constants α_0, α_1, and β_1 involve the characteristic

but unknown quantities D_{KG}, D_{H_2O}, Δx, and K, and must therefore be *evaluated from the experimental data. A method of evaluating these constants will be outlined in the next section.*

VIII

Evaluation of the Characteristic Constants

The data recorded by Osterhout were the volumes, V, in milliliters, of the aqueous phase C and the total salt normality in this phase for different values of the time, t, in hours. These data are recorded in the first three columns of Table I. Values of n_1, and n_0, which are recorded in Columns 5 and 6 of the table, were computed by means of the relations

$$n_1 = C \cdot V$$

$$n_0 = \frac{V \cdot d - 100.1\, n_1}{18.015}$$

100.1 and 18.015 being the molar weights of $KHCO_3$ and H_2O respectively. d is the density of the solution and was taken as equal to that of a pure aqueous solution of $KHCO_3$ as given in the International Critical Tables.[10] The values of the density are recorded in the fourth column.

The data of Table I may be used to evaluate the constants α_1, α_0, and β_1 as follows: Since equation (7.1) may be rearranged to

$$\frac{n_0 - I_0}{n_1} = \frac{n_0 - 3.321}{n_1} = \alpha_0(\beta_1 - \beta_0)\frac{t}{n_1} - \frac{\alpha_0}{\alpha_1}$$

a plot of $\dfrac{n_0 - 3.321}{n_1}$ as ordinate against $\dfrac{t}{n_1}$ as abscissae should give a straight line with a slope, $\alpha_0(\beta_1 - 0.00064)$, and an intercept, $-\alpha_0/\alpha_1$. This method of plotting the data (Fig. 2) magnifies small experimental errors in the initial stages of the experiment when n_0 differs but little from 3.321 and it is therefore not surprising that the first two points in Fig. 2 deviate considerably from a straight line. The remaining

[10] International Critical Tables, New York, McGraw-Hill Book Co., Inc., 1928, **3**, 90.

points yield a satisfactory straight line although such a line is not drawn in the figure. Since this plot is to be used for the evaluation of two of the constants which occur in the theory the final time curves for the entrance of salt and water can best be represented if all points in Fig. 2 are given equal weight. When this is done the straight line which best represents the data, as determined by the method of least

TABLE I

Experimental Data

1	2	3	4	5	6	7
t	$KHCO_3$ conc.	V	d^{25}_4 density	$n_1 = n^C_{KHCO_3}$	$n_0 = n^C_{H_2O}$	$\frac{n_1}{n_0} \simeq N_1 = N^C_{KHCO_3}$
hrs.	*mole/liter*	*ml.*				
0	0.00	60	0.997	0.0000	3.321	0 0000
16	0.10	60	1 004	0 0060	3.311	0.00181
42	0.26	64	1.014	0.01664	3.510	0.00474
65	0.40	68	1.023	0.0272	3.710	0.00733
80	0.46	72	1.027	0.03312	3.930	0.00843
104	0.53	76	1.032	0.04028	4.130	0.00975
128	0.60	81	1.037	0.0486	4.393	0 01106
151	0.61	87	1.037	0.05307	4.713	0.01126
178	0.62	93	1.038	0 05766	5.038	0.01145
208	0.63	99	1.038	0.06237	5.358	0.01164
232	0.63	105	1.038	0.06615	5.685	0.01164
256	0.63	112	1.038	0.07056	6.062	0 01164

squares, is the line drawn in the figure. The slope and intercept of this line furnish the relations

$$\alpha_0/\alpha_1 = 38.65 \tag{8.1}$$

$$\alpha_0(\beta_1 - 0.00064) = 0.02128 \tag{8.2}$$

A third relation between the three constants is necessary and may be obtained from the limiting slope of the n_0-t curve as the steady state is approached. The straight line of Fig. 3 appeared to have the correct slope, the value being

$$\left(\frac{dn_0}{dt}\right)_{\frac{n_1}{n_0} = 0.01164} = 0.01392 = \alpha_0(0.01164 - 0.00064) \tag{8.3}$$

Equations (8.1), (8.2), and (8.3) then give

$$\begin{aligned} \alpha_0 &= 1.265 \\ \alpha_1 &= 0.03273 \\ \beta_1 &= 0.01746 \end{aligned} \tag{8.4}$$

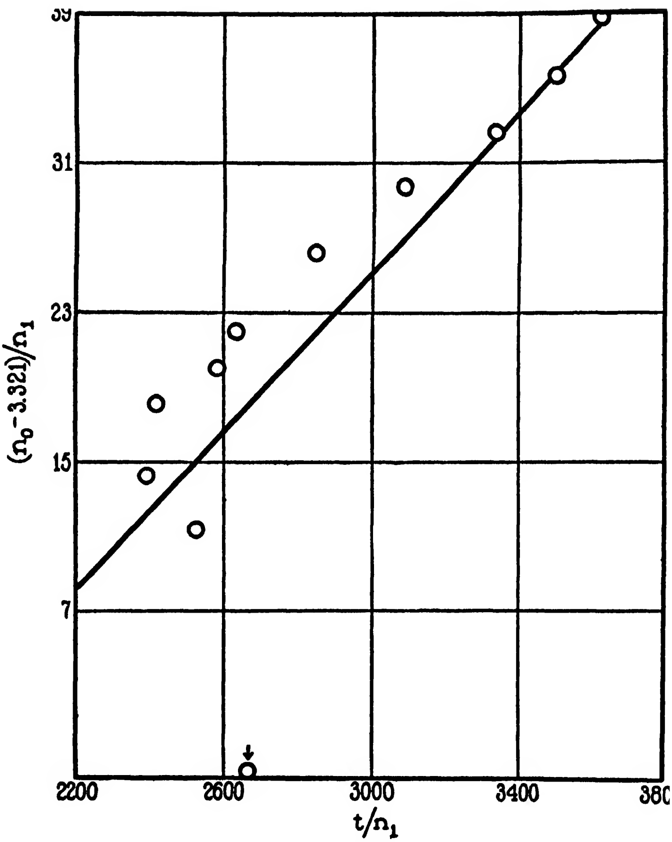

FIG. 2. A rectilinear plot of certain functions of the experimental data. From the slope and intercept of this line two of the three empirical constants which occur in the theory may be evaluated.

With these values of the constants the time curves may be computed by the method which has been outlined. The results of these computations are given in Table II and are plotted as smooth curves in Figs. 3 and 4.

TABLE II

Computed Values of n_0, n_1, and t

N_1	t	n_1	n_0
0.00064	3.77	0.0021	3.319_5
0.002	12.34	0.0066	3.327
0.004	26.80	0.0135	3.371
0.006	44.87	0.0208	3.471
0.008	70.09	0.0294	3.676
0.010	114.45	0.0415	4.152
0.0110	161.70	0.0522	4.745
0.0115	211.43	0.0623	5.414
0.01175	261.06	0.0717	6.104
0.0120	399.70	0.0970	8.079

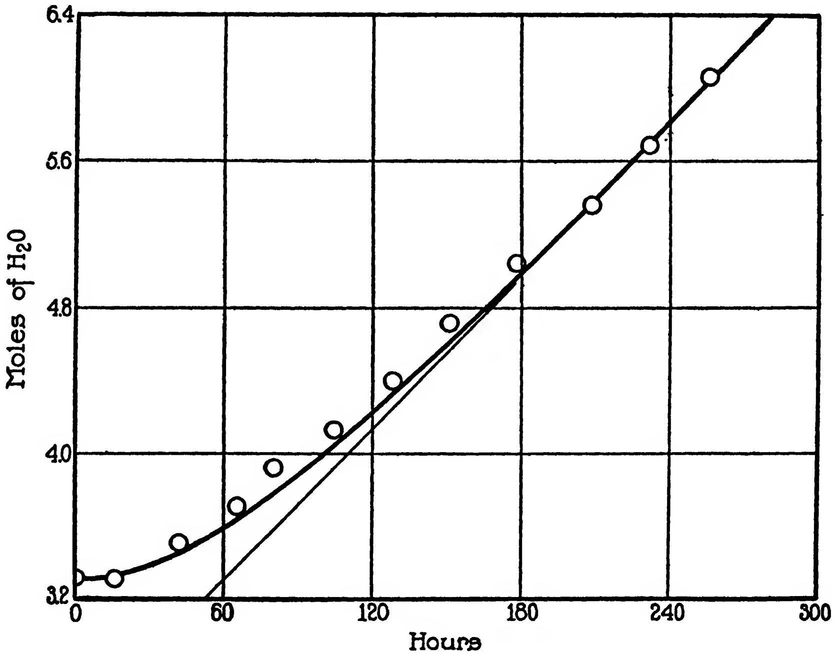

FIG. 3. A comparison of theory with experiment for the change of the water content of phase C with the time. The theoretical curve as computed from equation (7.8) is drawn as a full heavy line and the experimental points are indicated by the circles. The asymptote to this curve as the steady state is approached is drawn as a straight light line and the slope of this line was used in the evaluation of one of the constants of the theory

IX

Comparison of Theory with Experiment

The computed time curve for the entrance of water is in fair agreement with the experimental data which are plotted as circles in Fig. 3. Thus the theory predicts the minimum in this curve after the experiment has been in progress for a few hours. It will be recalled that this minimum occurs at the time that the water reverses its initial direction of flow and begins to move from A to C (Fig. 1). At this time

$$\frac{dn_0}{dt} = \alpha_0\left(-\beta_0 + \frac{n_1}{n_0}\right) = 0$$

and

$$\left(\frac{n_1}{n_0}\right) = \beta_0 = 0.00064$$

The values of n_0 and t corresponding to this value for $\dfrac{n_1}{n_0}$ are 3.319 and 3.77 respectively. Phase C thus has a minimum water content after about 4 hours.

Agreement with experiment in the case of the concentration-time curve is satisfactory as may be seen by reference to Fig. 4. An essential feature of the experiment—the approach to the steady state—is clearly illustrated. The characteristics of the steady state may be demonstrated as follows: Differentiation of the relation

$$N_1 = \frac{n_1}{n_0}$$

with respect to the time yields the expression

$$n_0 \frac{dN_1}{dt} + N_1 \frac{dn_0}{dt} = \frac{dn_1}{dt} \tag{9.1}$$

In the steady state N_1 is constant and equal to N_{1s}. Moreover $\dfrac{dN_1}{dt} = 0$ so that equation (9.1) becomes

$$N_{1s}\left(\frac{dn_0}{dt}\right)_s = \left(\frac{dn_1}{dt}\right)_s$$

From equations (6.10) and (6.11), however,

$$N_{1s}\,\alpha_0(-\,\beta_0 + N_{1s}) = \alpha_1(\beta_1 - N_{1s})$$

Solution of this quadratic gives, as the only physically possible root, the expression

$$N_{1s} = \frac{1}{2\alpha_0}\left(\alpha_0\beta_0 - \alpha_1 + \sqrt{(\alpha_1 - \alpha_0\beta_0)^2 + 4\alpha_0\alpha_1\beta_1}\right) \tag{9.2}$$

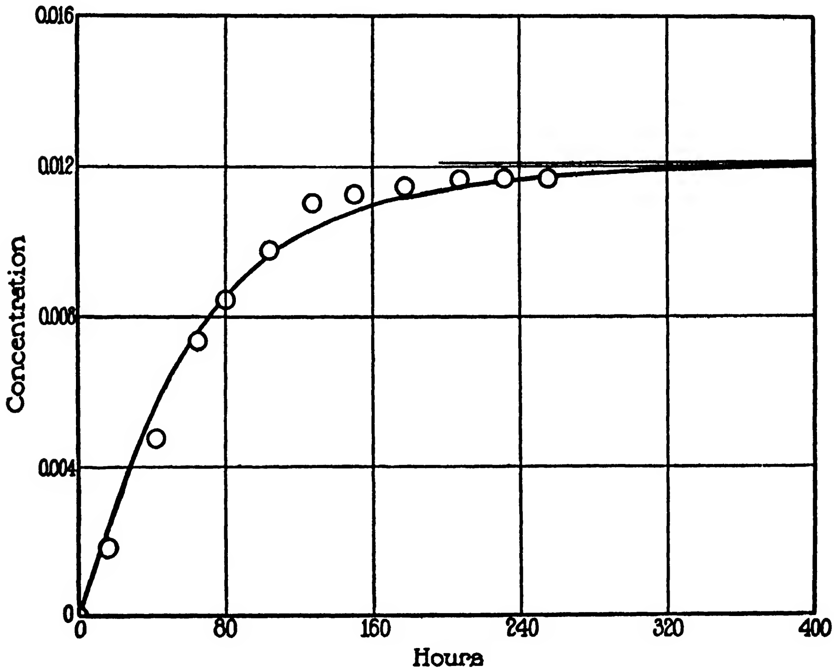

FIG. 4. A comparison of theory with experiment for the change of salt concentration in phase C with the time. The theoretical curve is drawn as a full heavy line and the experimental points are indicated by the circles. Concentrations are expressed as mole fractions and the light horizontal line is the theoretical value of the concentration in the steady state.

Substitution of numerical values for α_0, etc., leads to a value for N_{1s} of 0.0121 which is but slightly greater than the final values (0.011_6) which were observed during the latter stages of the experiment.

Through a combination of equations (9.2), (7.9), and (7.8) it may be shown that the steady state concentration is attained only after

infinite time, but a comparison of the curve of Fig. 4 with this limiting asymptote which is drawn as a straight horizontal line indicates that the concentration has reached a value only 5 per cent less than the limiting value after about 200 hours.

X

CONCLUSION

Some of the physical quantities which appear in the expressions for α_0, α_1, and β_1 (equations (6.6), (6.7), and (6.8)) are capable of independent measurement and in conclusion a discussion of these constants will be given. *Thus a value for K may be evaluated from certain properties of HG and H_2CO_3, namely, the solubilities of these* weak acids in water and their ionization constants.

In equation (2.5) both numerator and denominator may be multiplied by a_{H^+} to give

$$K = \frac{a_{HCO_3^-} \cdot a_{H^+}}{a_{G^-} \cdot a_{H^+}} \tag{10.1}$$

but $a_{HCO_3^-} \cdot a_{H^+} = K_{H_2CO_3} \cdot a_{H_2CO_3}$ and $a_{G^-} \cdot a_{H^+} = K_{HG} \cdot a_{HG}$ so that equation (10.1) may be written

$$K = \frac{K_{H_2CO_3} \cdot a_{H_2CO_3}}{K_{HG} \cdot a_{HG}} \tag{10.2}$$

Since $K_{H_2CO_3}$ has the value 4.54[11] $\times 10^{-7}$ and K_{HG} an even smaller value, $a_{H_2CO_3}$ may be replaced by the solubility of CO_2 at 1 atmosphere (0.034 mole/liter) and a_{HG} by the solubility of HG (0.15 mole/liter).

It is impossible to make an accurate estimation of K by means of equation (10.2) due to the fact that the weak acid represented by the symbol H*G* was, in the experiment of Osterhout and Stanley, a mixture of two weak acids, guaiacol (70 per cent) and *p*-cresol (30 per cent),[12] and also to the fact that the ionization constants for these substances

[11] MacInnes, D. A., and Belcher, D., *J. Am. Chem. Soc.*, 1933, **55**, 2630.

[12] In all computations involving this material, such as conversion of volume concentrations to mole fractions, etc., a molar weight, density, and molal volume of 119, 1.11, and 107, respectively, have been employed. These values are the means of the values for the pure substances, account being taken of their proportion by weight.

which are to be found in the literature are very discordant. Thus a hydrolysis method[13] gives

$$K(\text{guaiacol}) = 1.17 \times 10^{-10}$$

$$K(p\text{-cresol}) = 6.7 \times 10^{-11}$$

whereas a conductance method[14] gives

$$K(p\text{-cresol}) = 1.1 \times 10^{-8}$$

If guaiacol and *p*-cresol are assumed to have essentially the same ionization constants, as the results of hydrolysis measurements would indicate, K in equation (10.2) has the value 9×10^2 if 1.1×10^{-10} is assumed for K_{HG} and the value 9 if $K_{HG} = 1.1 \times 10^{-8}$. The value of 21 which may be computed from equation (6.8) and which best fits the diffusion data is intermediate between the two independently computed values. The results of this computation are inconclusive except in so far as the independent estimate of K indicates that most of the KG which enters C is converted into $KHCO_3$.

From equations (6.6) and (6.7) it is evident that

$$\frac{AD_{KG}}{V_{HG}\Delta x} = \frac{\alpha_1 K}{S^b_{KG}} = 0.37$$

$$\frac{AD_{H_2O}}{V_{HG}\Delta x} = \frac{\alpha_0}{S_{H_2O} \cdot i^C} = 1.8$$

The ratio of the two diffusion coefficients is $D_{H_2O}/D_{KG} = 4.8$, a value which indicates that H_2O diffuses through the non-aqueous layer much more rapidly than KG. This ratio is somewhat higher than would be expected from a consideration of molecular size and may be due to the fact that the concentration of water in the non-aqueous phase is so high that certain assumptions which were made in the derivation of the diffusion equations are partially invalidated.

Osterhout, Kamerling,[4] and Stanley have shown that the diffusion

[13] Landolt, H., and Börnstein, R., Physikalisch-chemische Tabellen, Berlin, Julius Springer, 5th edition, (Roth, W. A., and Scheel, K.), 1931, suppl. vol. **2**, pt. 2, pp. 1087–88.

[14] International Critical Tables, New York, McGraw-Hill Book Co., Inc., 1929, **6**, 281.

of K*G* in H_2O is about twelve times as rapid as in H*G*. The value of D_{KG} for diffusion in water is not known but it will certainly be of the same order of magnitude as the diffusion coefficient for a potassium salt with a large organic anion such as potassium acetate, namely, 1×10^{-5} $\overline{\text{cm.}}^2$/second. Thus D_{KG} for diffusion in H*G* is of the order 1×10^{-6} $\overline{\text{cm.}}^2$/second or 3.6×10^{-3} $\overline{\text{cm.}}^2$/hour. The mean area of the two phase boundaries *a* and *b* (Fig. 1) was about 95 $\overline{\text{cm.}}^2$. Consequently

$$\Delta x = \frac{AD_{KG}}{0.37\ V_{HG}} = \frac{95 \times 3.6 \times 10^{-3}}{0.37 \times 107} = 0.01 \text{ cm.}$$

From direct microscopic observation Davis and Crandall[15] have estimated that the thickness of the unstirred water layer at a gas-water interface is about 0.04 cm. While the two values are by no means comparable it appears that the value for Δx which has just been computed is a physically possible one. It is worthy of note that this value is sufficiently low to justify the assumption of a linear concentration gradient in the unstirred layers which was made.

The theory which has been developed in this paper is subject to many obvious refinements and it may be necessary to amend certain aspects of the physical interpretation of the experiment. Thus there are many[16] who consider reaction velocities in heterogeneous systems to be determined by a slow attainment of equilibria at the phase boundaries and not by the time element in the diffusion across unstirred layers. It is important to note, however, that either picture will lead to essentially the same differential equations as those developed in this paper though, of course, the physical interpretation of the constants will differ.

XI

SUMMARY

The differential equations which describe the simultaneous diffusion of water and a salt in a cell model have been formulated and solved. The equations have been derived from the general laws which describe

[15] Davis, H. S., and Crandall, G. S., *J. Am. Chem. Soc.*, 1930, **52**, 3757.
[16] See, for example, Roller, P. S., *J. Phys. Chem.*, 1932, **36**, 1202.

diffusion processes, thereby furnishing a physical interpretation for the constants which enter into the theory. The theoretical time curves for the two diffusing substances are in good agreement with the experimentally determined curves and accurately reproduce all of the essential characteristics of the experiment.

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THE ACTION OF CARBON MONOXIDE ON IRON AND COBALT COMPLEXES OF CYSTEINE

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I. Introduction.—In continuation of studies of the iron and cobalt complexes of thiol acids, the reaction of carbon monoxide with these compounds has been taken up. Cremer[1] was the first to observe that solutions of ferrous and cobaltous complexes of cysteine absorb carbon monoxide and from a study of the maximum amount absorbed and the proportion of metal to cysteine under which this takes place, concluded that there must exist in solution the complexes Fe(cysteine)$_2$(CO)$_2$ and Co(cysteine)$_2$(CO). The concern of the present paper is the actual isolation of the complexes which occur in solutions of ferrous and of cobaltous biscysteinate after they have absorbed carbon monoxide.

II. The Ferro Biscysteinate Dicarbonyl Complex.—A solution of potassium ferro biscysteinate, whose isolation has been described previously,[2] was made by mixing the components in the proportions of two moles of cysteine hydrochloride, one mole of ferrous salt and six moles of potassium hydroxide. This amount of alkali is just equivalent to the sum of the iron and acid. Such a solution made in an atmosphere of carbon monoxide absorbs two moles of the gas in two to four hours after which there is no further absorption. The data need not be given as they agree with those of Cremer. Even with double this proportion of alkali the total amount of carbon monoxide absorbed does not change though the rate of absorption is lower. This behavior differs markedly from that of the cobalto biscysteinate complex as will be shown later.

The complex can easily be prepared from its components provided oxygen be carefully excluded during its formation. For this purpose a special flask was constructed. A 250-cc. Erlenmeyer flask is fitted with a side arm having a glass stop-

(1) W. Cremer, *Biochem. Z.*, **206**, 228 (1929).

(2) Schubert, THIS JOURNAL, **54**, 4077 (1932).

cock. Into the neck of the flask is ground a glass stopper carrying a gas inlet tube also fitted with a glass stopcock and a small dropping funnel. In the flask are placed 3.9 g. of $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$, 3.2 g. of cysteine hydrochloride and 20 cc. of water. The air is swept out with hydrogen freed of oxygen by passage over heated copper. The test described by Von Wartenberg,[3] the disappearance of the phosphorus halo, is used to show when oxygen has been removed. Then 8 cc. of 6.5 *M* potassium hydroxide is admitted through the dropping funnel and the orange color of the ferro biscysteinate complex appears. A stream of carbon monoxide, generated from formic and sulfuric acids and washed with alkaline pyrogallol, is passed through the flask while the mixture is shaken steadily for four hours. The color appears to deepen during this process. Ten cc. of concentrated hydrochloric acid is added, the mixture shaken and set on ice. Precautions against admission of oxygen are no longer necessary as the carbon monoxide complex is stable in air. The mixture rapidly becomes solid with masses of yellow needles. These are filtered off, washed with a little ice cold water and alcohol and dried in a desiccator over sulfuric acid at 1 mm. There is no tendency for carbon dioxide to dissociate in a vacuum as determined by analysis after leaving *in vacuo* for various periods of time.

Anal. Calcd. for $Fe(SCH_2CHNH_2COOH)_2 \cdot 2CO \cdot H_2O$: Fe, 15.13; S, 17.29; N, 7.57; C, 25.94; H, 3.82. Found: Fe, 15.49; S, 17.28; N, 7.70; C, 26.15; H, 3.86.

The sodium salt can be prepared using sodium hydroxide in place of potassium hydroxide and of course omitting the addition of acid. On cooling in an ice-box overnight fine needles separate. After separating, washing and drying in a vacuum they show the following analysis.

Anal. Calcd. for $Fe(SCH_2CHNH_2COONa)_2 \cdot 2CO \cdot 2H_2O$: Fe, 12.96; S, 14.81; Na, 10.65; C, 22.21; H, 3.27. Found: Fe, 13.32; S, 15.18; Na, 10.61; C, 22.08; H, 3.93.

These experiments were carried out in diffuse daylight and it made no apparent difference in the amount of carbon monoxide absorbed whether the contents of the flask were protected from such light or not. This need not contradict Cremer's observation that the complex dissociates carbon monoxide on illumination as no experiments were carried out under intense illumination. Both of these compounds are quite stable in air when dry, the free acid decomposes in solution, within a few days at ice temperature and within an hour on warming. This decomposition is simply a dissociation, the total carbon monoxide being liberated as such and the ferro biscysteinate complex breaking up to ferrous salt and cysteine. As for the structure of this complex there seems to be no reason at present for supposing it other than the same as potassium ferro biscysteinate[2] with two carbonyl groups held by coördinate links directly to the iron:

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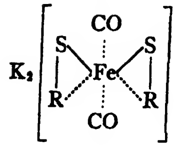
(3) H. Von Wartenberg, *Z. Elektrochem.*, **36**, 295 (1930).



where R = —CH_2CHNH_2COO—

III. The Action of Carbon Monoxide on Potassium Cobalto Biscysteinate.—Measurements of the amount of carbon monoxide absorbed by potassium cobalto biscysteinate were made as described for the corresponding ferro complex. With the proportions of one mole of cobalt chloride, two of cysteine hydrochloride and six of potassium hydroxide the carbon monoxide absorbed amounted to about one mole in four hours after which there was no further absorption. The data for this are given in Table I, row 2, marked "equiv." But in these mixtures no complex at all analogous to the ferro biscysteinate dicarbonyl just described could be found. Two complexes of cobalt were, however, isolated, of which one contains only cysteine and the other only carbon and oxygen. The first, an oxidation product, is simply the green potassium cobalti triscysteinate, $K_3[Co(RS)_3]$ where R is the divalent radical (—CH_2CHNH_2COO—). The preparation of this complex from cobalt salt and cysteine has already been described.[4] The second complex could only be isolated as the silver and mercuric derivatives whose composition agrees reasonably with the formulas $Ag[Co(CO)_4]$ and $Hg[Co(CO)_4]_2$. In the flask described in the last section are placed 5 g. of cysteine hydrochloride and 15 cc. of 1 *M* cobalt chloride. After the air is completely displaced, 15 cc. of 6.3 *M* potassium hydroxide is added. Carbon monoxide is passed through while the mixture is shaken for four hours. At this point there always remains a small deposit of cobalt hydroxide.

To separate the green potassium cobalti triscysteinate and at the same time to show that its formation is not due to air oxidation occurring during its isolation, the stopcocks on the flask are closed and the whole flask set in the tank described previously[4] for the separation of complexes in the absence of air. After the air has been completely displaced from the tank by nitrogen and hydrogen, the flask is opened, 50 cc. of alcohol added to the contents and the mixture shaken. After an hour the mixture is filtered on a Buchner funnel, the solid dissolved in 40 to 50 cc. of water and reprecipitated with an equal volume of alcohol. After filtering, washing and drying in a vacuum, between 4 and 5 g. of product is obtained.

Anal. Calcd. for $K_3[Co(SR)_3]\cdot 3H_2O$: Co, 10.05; S, 16.35; N, 7.16; K, 19.93. Found: Co, 9.66; S, 16.02; N, 6.92; K, 20.41.

To show the presence of the second complex the following procedure was found best. To the mixture described above, after the absorption of carbon monoxide, there is added 100 cc. of acetone. After fifteen minutes the whole is filtered, and the filtrate quickly evaporated *in vacuo* to about 15 cc. To this is added a solu-

(4) Schubert, This Journal, 55, 3336 (1933).

tion of a gram of mercuric chloride in 20 cc. of water and then after a few minutes 15 cc. of 6 *M* hydrochloric acid. A yellow, more or less crystalline precipitate is obtained which is washed with dilute hydrochloric acid and then water. It can be recrystallized easily by dissolving in a small volume of acetone, filtering if necessary and then adding two volumes of water. The precipitate slowly crystallizes to yellow needles or rectangular yellow plates. It is dried *in vacuo* and protected from light; yield 0.5 g.

Anal. Calcd. for $Hg[Co(CO)_4]_2$: Hg, 37.00; Co, 21.78; C, 17.71; H, none. Found: Hg, 37.37, 37.52; Co, 21.16, 21.51; C, 17.38; H, 0.25.

This mercury salt is quite stable. It darkens when exposed to sunlight. It is very insoluble in water and non-oxidizing acids but dissolves readily in alcohol, ether, chloroform and acetone. It melts without apparent decomposition at 82°.

To separate the silver salt, the procedure given above for the isolation of the mercury salt is followed as far as the evaporation of the acetone *in vacuo*. To the clear solution remaining after this evaporation there is added 5 cc. of concentrated ammonium hydroxide and as much of a solution of 2 g. of silver nitrate in 10 cc. of water plus 5 cc. of concentrated ammonium hydroxide as is necessary to cause complete precipitation. The precipitate, at first liquid, crystallizes rapidly. This is filtered off, washed with dilute ammonia, then with water, and dried in a desiccator over concentrated sulfuric acid at 1 mm. The product is light yellow and consists of needle-like crystals. It is not very stable, especially in air or when exposed to light, turning first gray, then black within an hour after its separation from solution. For this reason the analyses here given were run on samples which had been left in the desiccator only ten minutes. The water is probably residual moisture and the formula is not to be understood as implying the existence of a hydrate.

Anal. Calcd. for $Ag[Co(CO)_4] \cdot \frac{1}{2}H_2O$: Ag, 37.45; Co, 20.48; C, 16.66; H, 0.35. Found: Ag, 36.32; Co, 20.20; C, 16.42; H, 0.2.

Another preparation was apparently wetter.

Anal. Calcd. for $Ag[Co(CO)_4] \cdot 2H_2O$: Ag, 34.21; Co, 18.74; C, 15.42; H, 1.27. Found: Ag, 32.42; Co, 19.26; C, 14.89; H, 1.29.

This silver salt can be recrystallized from acetone just like the mercury salt. Its solubilities are similar to those of the mercury salt but it has no melting point, merely turning black when heated to about 90°. The yield of silver salt after recrystallization is about 400 mg.

IV. The Action of Carbon Monoxide on Potassium Cobalti Triscysteinate.—If larger quantities of potassium hydroxide are used in the experiment described in the last section, the absorption of carbon monoxide does not reach an end-point even after twenty-four hours and the amount of carbon monoxide absorbed in this period increases with the amount of potassium hydroxide used, as shown in Table I. In casting about for a possible explanation of this remarkable phenomenon it

was found that potassium cobalti triscysteinate, one of the products of the reaction described in the last section, will itself absorb large quantities of carbon monoxide when in strongly alkaline solution and that this absorption is much slower than that discussed in the previous section, requiring twenty-four to forty-

TABLE I

Millimoles of carbon monoxide absorbed by a mixture of 3.2 g. (20 mml.) of cysteine hydrochloride, 9.6 cc. of 1 *M* $CoCl_2$ solution and the number of cc. of 6.3 *M* KOH solution shown in column 1. The total solution volume is made up to about 40 cc. with water. Temperature 30°. The mixtures were shaken mechanically for the time indicated.

Cc. of 6.3 KOH *M*	Millimoles of CO absorbed after the indicated number of hours					
	1	2	4	8	24	32
7.1	4.2	5.5	6.1	6.9	7.5	..
9.5 (equiv.)	7.8	9.0	9.4	9.5	9.7	9.7
10.0	8.3	11.0	12.1	12.2	12.4	12.3
15	3.6	6.9	10.8	15.8	25.9	27.8
20	3.5	7.0	12.0	19.1	34.9	36.3
25	4.7	9.0	13.2	22.4	38.5	42.0
30	3.8	7.6	12.3	21.7	39.6	43.2

TABLE II

Millimoles of carbon monoxide absorbed by a solution of 1 g. of the green potassium cobalti triscysteinate in 10 cc. of water with the addition of the number of cc. of 6.3 *M* KOH shown in column 1. One gram of this complex is 1.7 millimoles. The mixtures were shaken mechanically for the time indicated. Temperature, 30°

6.3 *M* KOH, cc.	Millimoles of CO absorbed after the indicated number of hours					
	1	2	4	8	24	32
0	0	0	0	0	0	0
1	0.2	0.6	1.7	3.3	5.1	5.3
2	.2	.5	1.6	3.8	7.1	7.6
3	.4	1.0	2.8	5.0	7.6	8.1
4	.4	1.3	3.6	6.9	8.9	9.0

eight hours to approach completion. Table II shows that with a sufficient amount of alkali, one mole of this cobalti triscysteinate will absorb as much as five to six moles of carbon monoxide. One of the products of this reaction is potassium carbonate which was separated as barium carbonate while the other product is the

same new complex described above, $Hg[Co(CO)_4]_2$, again separated as silver and mercury derivatives. In this reaction as in the preceding there again appears an oxidation product, the carbonate which could only have come from the carbon monoxide used.

The volume of carbon monoxide consumed was measured in a 500-cc. suction flask connected to a calibrated gas reservoir provided with a leveling tube and an outlet. In the bottom of the flask were placed small beakers containing the components of the mixtures described in Tables I and II. After sealing the connections with paraffin, carbon monoxide is passed into the suction flask, out the side arm through the gas reservoir, finally escaping through the outlet of the latter. When all the air has been displaced the whole apparatus is closed off by stopcocks and set on a shaking machine in a room kept at 30 ± 0.2°. When the system has come to temperature equilibrium the volume of gas in the reservoir is read, the beakers in the flask upset, mixing the components, and the shaking machine started.

To carry out the reaction described in this section, 5 g. of potassium cobalti triscysteinate, prepared as described elsewhere,[4] and 15 cc. water are placed in the flask described in the preceding section. After air has been removed by carbon monoxide 20 cc. of 6.3 *M* potassium hydroxide, freed of carbonate by saturation with barium hydroxide, is added. The flask is then connected to a calibrated reservoir of carbon monoxide under slightly more than atmospheric pressure and shaken for about forty hours. Forty-five millimoles of carbon monoxide are absorbed. The very dark green color of the solution disappears in this time, only a light brown clear solution remaining. To separate the carbonate formed this solution is diluted with some water and about 25 cc. 1 *M* barium chloride added. The precipitate is filtered off, washed and dried and can be shown by a few simple tests and by barium analysis to be practically pure barium carbonate. Three and one-tenth grams is obtained.

The silver and mercury salts of the complex $H[Co(Co)_4]$ can be separated by extraction of the solution resulting from the carbon monoxide absorption with three 80-cc. portions of acetone and treatment of this acetone extract as already described. Two grams of the silver salt or 1.7 g. of the mercury salt is recovered after purification by recrystallization from acetone by addition of water. The properties and analyses of these two salts agree completely with those resulting from the reaction described in the preceding section.

V. The Formation of Cobalt Tetracarbonyl.—If either of the reaction mixtures produced by the absorption of carbon monoxide, as described in the last two sections be diluted with two volumes of water and then acidified with hydrochloric acid, some gas is liberated and an extremely choking odor is produced strongly suggestive of impure acetylene. At the same time a yellow precipitate of plate-like crystals forms, extremely insoluble in water, dilute acids or alkalies. These are filtered off, washed with much water and set in a vacuum desiccator. They are very unstable in a dry state, so, like the silver salt described above, they must

be analyzed after only a few minutes drying. Like the silver salt, they fortunately dry very quickly. The analysis and properties of these crystals correspond to those of cobalt tetracarbonyl.

Anal. Calcd. for $Co(CO)_4 \cdot {}^1/_2H_2O$: Co, 32.79; C, 26.67; H, 0.56. Found: Co, 32.70, 32.38; C, 25.88, 26.07; H, 0.48, 0.52.

The water in the formula is probably simply a residue of moisture and is calculated in this way merely for convenience. The yellow crystals are very unstable in air, usually turning black within an hour after their separation in a dry condition. They are volatile and have been sublimed *in vacuo*. No melting point could be obtained as the crystals sinter and decompose at 48 to 50° even under nitrogen. They dissolve readily in alcohol, ether, chloroform and benzene and such solutions show a slow evolution of gas and soon deposit a black amorphous precipitate. The crystals even after long washing with water persist in emitting traces of the choking acetylenic odor mentioned above and it cannot be decided at present whether this is the odor of the crystals themselves or of a product of their decomposition.

If the mixture described in section III be used to prepare the carbonyl a yield of 200 to 250 mg. is obtained, while from the mixture described in section IV 700 mg. results. Made from the mixture of section IV the carbonyl is never as pure as when made from the mixture of section III. This may be due to the fact that the reaction of section IV requires forty hours in the presence of strong alkali so that some decomposition or secondary reaction occurs, making it impossible to obtain as pure a product as from the mixture of section III where the conditions are milder.

VI. The Reaction of Cobalt Tetracarbonyl with Cysteine.—A half gram of freshly prepared cobalt tetracarbonyl is added to a solution of 2 g. of cysteine hydrochloride in 15 cc. water, 6 cc. 6.3 *M* potassium hydroxide and 15 cc. alcohol. The alcohol must be added as the carbonyl is too insoluble in water for any reaction to occur. With this mixture the carbonyl slowly goes into solution and at the same time a gas is evolved; 2.2 millimoles of gas are evolved, all of which is absorbed by ammoniacal cuprous chloride. The solution itself slowly deposits the characteristic green crystals of potassium cobalti triscysteinate even when the whole reaction is conducted under nitrogen in the tank used for the preparation of oxygen-sensitive compounds. Under these conditions 0.33 g. of the green complex have been obtained after recrystallization from water by addition of alcohol. Finally there was separated by the method described in section III, between 0.4 and 0.5 g. of the compound $Hg[Co(CO)_4]_2$.

VII. Discussion of Results.—The mixture discussed in section III has been shown[4] to give rise to the following reaction in the absence of air: $CoCl_2 + 2HSRH \cdot HCl + 6KOH \rightarrow K_2[Co(SR)_2] + 4KCl + 6H_2O$ where R has the meaning defined in section III. When carbon

monoxide is passed through this solution, it is quite possible that the first reaction is the same as that described in section II for the corresponding iron complex where potassium ferro biscysteinate dicarbonyl is the product. But whereas the iron complex is stable in this ferrous state the cobalt in the analogous hypothetical complex, having a greater tendency to assume the trivalent condition, does so at the expense of reducing some other reactant. The equation for the total reaction can be written

$$9K_2[Co(SR)_2] + 8CO + 2H_2O \rightarrow 6K_3[Co(SR)_3] + 2H[Co(CO)_4] + Co(OH)_2 \quad (1)$$

The formation of carbonate in the reaction of section IV means that this is also an oxidation–reduction reaction. It can be represented thus

$$K_3[Co(SR)_3] + 6CO + 7KOH \rightarrow H[Co(CO)_4] + 2K_2CO_3 + 3K_2(RS) + 3H_2O \quad (2)$$

The assumptions and reasoning made use of for arriving at equations (1) and (2) cannot be given here for lack of space but a study of the data given in sections III and IV will show that all the products and only those demanded by these equations were actually found. Furthermore, the quantities of reactants and products will be found to be in fair agreement with these equations particularly considering that wherever possible products were purified by recrystallization.

No adequate formulation of the reaction described in section V can be given as it has been impossible so far to account for the hydrogen which it has been assumed occurs in the cobalt tetracarbonyl hydride. When this compound is, in part at least, converted to cobalt tetracarbonyl either free hydrogen or some reduction product, possibly of carbon monoxide, would be expected to result but none such could be found.

Finally, the following equation agrees very well both qualitatively and quantitatively with the products found for the reaction of section VI.

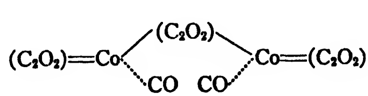
$$4Co(CO)_4 + 3H_2RS + 3KOH \rightarrow 3H[Co(CO)_4] + K[Co(RS)_3] + 4CO + 3H_2O \quad (3)$$

Although equations (1), (2) and (3) seem to summarize correctly the experimental findings, there remains the ambiguity attaching to the unformulated constitution of $H[Co(CO)_4]$. An assumption that

has been found useful as a working hypothesis in dealing with these unusual reactions is that in the formation of the new complex the carbon monoxide has been reduced to a divalent negative radical $(C_2O_2)^{=}$. The only two plausible structures which can be suggested for this radical are $(-OC \equiv CO-)$ and $(-\underset{\underset{\textstyle O}{\|}}{C}-\underset{\underset{\textstyle O}{\|}}{C}-)$. The first has already been suggested by Reihlen[5] in his studies on iron carbonyls and their derivatives. The second has been suggested by Joannis,[6] who isolated the compound $K_2C_2O_2$ by the action of carbon monoxide on a liquid ammonia solution of potassium.

On this view the new complex would be a cobalti complex of a divalent pseudo acid radical, $H[Co(C_2O_2)_2]$ which in alkaline solution might exist as a potassium salt. This suggests the interpretation of the oxidation-reduction reactions (1) and (2). In fact if the essential part of (2) is written alone, we have: $3CO + 4(OH)^- \rightarrow (C_2O_2)^{=} + CO_3^{=} + 2H_2O$. It thus resembles a Cannizzaro reaction on a free carbonyl group occurring appropriately in strong alkali.

Cobalt carbonyl has usually been regarded like most of the metal carbonyls as being a pure coördination compound, that is, as being composed of zero valent cobalt and coördinatively bound carbon monoxide. Reaction (3) which can be carried out completely in the absence of air and in which no stronger oxidant than water is present gives as products one complex, the cobalti triscysteinate, in which the cobalt is certainly trivalent and another in which it is probably trivalent. With this reaction in mind it seems difficult to regard the cobalt in the carbonyl as zero valent. The following structure involving trivalent cobalt would be much more in agreement with the experiments described here.



This formula in addition to accounting for reaction (3) would explain why cobalt tetracarbonyl is dimolecular and why it loses two moles of carbon monoxide rather easily to form cobalt tricarbonyl.

(5) Reihlen and co-workers, *Ann.*, **465**, 72 (1928); **472**, 268 (1929).
(6) M. A. Joannis, *Compt. rend.*, **116**, 1518 (1893); **158**, 874 (1914).

Hieber[7] describes a reaction of acid with tricarbonyl-*o*-phenanthroline cobalt among the products of which is cobalt tetracarbonyl and a carbonyl compound with hydrogen attached directly to the cobalt, $[Co(CO)_4H]$, as well as a volatile unstable cobalt compound with an evil odor. *The cobalt carbonyl hydrogen compound, for whose existence Hieber defers giving any evidence, might be one assumed here from which the mercury and silver compounds are derived.*

Hieber[8] has also described a reaction of $Fe(CO)_5$ in strong alkali which bears some resemblance to the reaction of section IV. He formulates it as: $Fe(CO)_5 + 2(OH)^- \rightarrow [Fe(CO)_4H_2] + CO_3^{=}$. The hydrogen in the iron carbonyl hydride is regarded as being attached directly to the iron by a peculiar kind of bond.

Further study of these compounds would no doubt add to our knowledge of valence.

Mr. G. Bitterlich of this Laboratory carried out all the analytical work described here.

SUMMARY

The complex ferro biscysteinate dicarbonyl has been crystallized as a free acid and as its sodium salt. The action of carbon monoxide on potassium cobalto biscysteinate has been shown to involve oxidation and reduction, the products being the green potassium cobalti triscysteinate and a new complex, isolated as the silver and mercury compound, apparently derived from $H[Co(CO)_4]$. The action of carbon monoxide on the green potassium cobalti triscysteinate in strong alkali also involves oxidation and reduction, the products being carbonate and the new complex $H[Co(CO)_4]$. The complex $H[Co(CO)_4]$, on treatment with acid, gives cobalt tetracarbonyl. Cobalt tetracarbonyl on treatment with cysteine and alkali gives potassium cobalti triscysteinate and $H[Co(CO)_4]$.

(7) W. Hieber, F. Muhlbauer and E. A. Ehmann, *Ber.*, **65**, 1090 (1932).
(8) W. Hieber and F. Leutert, *Z. anorg. allgem. Chem.*, **204**, 155 (1932).

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THE EFFECT OF IRON ON THE ESTABLISHMENT OF THE OXIDATION-REDUCTION POTENTIAL OF ALLOXANTIN

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On considering the various instances where heavy metal salts act as catalysts in oxidation-reduction processes, the following observation seems to be of interest in which iron acts as a catalyst for the establishment of an oxidation-reduction potential.

Biilmann and Lund[1] have shown that an acidified solution of alloxantin establishes a definite potential at the blank platinum or gold electrode. The interpretation is based on the well-founded assumption that alloxantin in an aqueous solution is split into dialuric acid and alloxan and that these two substances behave as the components of a reversible oxidation-reduction system. The case of alloxantin is thus analogous to that of quinhydrone. Richardson and Cannan[2] extended the potentiometric study over a wide pH range and performed oxidative potentiometric titration experiments which, in spite of difficulties with respect to the lack of stability of the potentials, led to reasonable results.

The reproduction of Biilmann's experiment is easy, provided one works with rather concentrated solutions of alloxantin (about 0.3 per cent.), best in saturated solution with an excess of solid alloxantin. In this case, of course, oxidative or reductive potentiometric titration experiments can not be performed.

On the occasion of our own potentiometric study of alloxantin it appeared to us striking that the potentials become erratic, inconstant in time and irreproducible, when a somewhat diluted solution of alloxantin is used instead of a saturated one, a fact which has never been mentioned anywhere. A further study showed that on the addition of a small amount of an iron salt the potentials become reproducible

[1] E. Biilmann and H. Lund, *Ann. Chim.* (9) 19: 137, 1923.

[2] G. M. Richardson and R. K. Cannan, *Biochem. Jour.*, 28: 68, 1929.

and are rapidly established, even in very dilute solutions of alloxantin (1:10,000 and even more dilute). Mere traces of iron are not sufficient to establish the full effect, but well-measurable amounts are requisite. About 0.1 to 1.0 milligram of iron, as sulphate, added to a volume of 25 cc. of the solution is necessary. On the other hand, in a concentrated solution of alloxantin, Fe is not requisite. On working with all precautions as Fe-free as possible and adding such powerful Fe-combining reagents as alpha, alpha'-dipyridyl, or alpha, alpha'-phenantrolin the potentials are quickly established in concentrated solutions.

Alloxantin can be reduced in the electrode vessel by hydrogen and colloidal palladium to dialuric acid, and after replacing the hydrogen by nitrogen an oxidative titration experiment with bromine can be performed. The oxidation product is alloxan. The establishment of the potentials is sluggish and erratic. When, however, a small amount of $FeSO_4$ had been added to the mixture, the potentials are immediately established, just as with any reversible dyestuff. The shape of the titration curve is precisely the one of an ordinary organic dyestuff system, with no indication of any intermediary step of oxidation being shown. This intermediary form, known as alloxantin in the crystalline state, does not exist to any appreciable extent in the solution, just as the substance called quinhydrone in its crystalline state does not exist in solution. The titration curve is not influenced at all by the adding even of a great excess of iron.

The effect of the iron is most evident between pH 4 and 6. At lower pH the effect is small and at pH about 1.0, where Biilmann and Lund worked, no appreciable effect of iron can be seen any more. At so low a pH only a concentrated solution of alloxantin establishes a reproducible potential, and this is not influenced at all by iron.

Iron can not be replaced by copper, manganese, cobalt, nickel or organic dyestuffs.

As for the explanation of this effect, it should be recalled that dialuric acid gives a complex compound with iron. This can be shown, however, only in an alkaline solution. Dialuric acid plus $FeCl_3$, plus ammonia, produces a deep violet color. At the pH range 4 to 6, where the effect of iron on the potential is strongest, no evidence of any complex formation can be shown. No color and no precipitate is produced by the iron. If there exists, at pH 4–6, a Fe complex at all, this com-

plex can represent only a very slight fraction of that part of the substance not combined with Fe. Yet, this minute trace of the complex may be considered as the intermediator for the establishment of the potential. Without emphasizing too much this hypothesis, at any rate, this is another case of the catalytic establishment of a potential in a system which is thermodynamically reversible but sluggishly reactive without a catalyst.

It should be recalled[3] also that Fe acts as a catalyst for the oxidation of dialuric acid by molecular oxygen. The pH optimum of this effect is around pH 7, whereas the effect described above has its optimum at pH 4–6.

[3] Edgar S. Hill, *Jour. Biol. Chem.*, 92: 471, 1931.

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THE ACTION OF TYPE-SPECIFIC HEMOPHILUS INFLUENZAE ANTISERUM

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In a previous study (1) of type-specific strains of *Hemophilus influenzae*, it was observed that the organisms of all the strains isolated from cases of "influenzal meningitis" were encapsulated, formed colonies which have been described as smooth, and produced a soluble specific substance which precipitated the homologous antiserum. At the time the above report was made, organisms from nine patients not closely associated had been studied, and all of the strains were similar and belonged to the group which has been designated as Type b. This suggested that most, or at least the majority of, meningitis strains were of the same serological type.

In 1922, Dr. T. M. Rivers concentrated broth cultures of two meningitis strains and then removed from the concentrates the alcohol-precipitable fractions. 9 years later, these fractions were given to the author to be tested immunologically. Solutions of the fractions from both strains were made, and in each solution a heavy precipitate was formed when Type b antiserum only was added. This indicated, therefore, that the two strains studied by Dr. Rivers were also of the Type b group.

It was mentioned previously (1) that Wollstein (2), Povitsky and Denny (3), Rivers and Kohn (4), and others, found a considerable degree of immunological relationship among the strains isolated from cases of meningitis. There may be some question, however, whether the relationship which they noted was that based on the possession of the common soluble specific substance which we have described. Many of the strains had been isolated a number of months before they were immunologically tested, and as organisms of the S form usually tend to change to those of the R form with great rapidity, it is probable that some of their cultures contained only R forms. Furthermore, the agglutination reactions studied by these observers were carried out at high temperatures (45°C. or higher), under which conditions type-specific reactions are at least partially masked.

Ward and Wright (5), and Ward and Fothergill (6), have also reported that they have observed a close immunological relationship between meningitis strains.

Our observation that the majority of meningitis strains are type-specific and of the same type has been further substantiated in the continuation of this study. Up to the present, 41 strains of Pfeiffer bacilli obtained from patients suffering from lesions of the central nervous system have been examined, and all but four have been of Type b.[1] Three of these exceptions will be discussed later, and in the fourth instance the bacterium isolated was not of the species *Hemophilus influenzae*, but of the species *Hemophilus parainfluenzae*. This organism was isolated by Dr. J. D. Trask from the spinal fluid of a child who had a brain abscess.

The discovery that the organisms present in influenzal meningitis are usually type-specific, and that most of them are immunologically identical, suggested that a highly immune horse serum might have therapeutic value. Earlier attempts by other investigators to treat influenzal meningitis with immune serum were not very successful.

Wollstein (7), and also Rivers (8), did not find *H. influenzae* antiserum very effective in treating cases of influenzal meningitis in children, although Wollstein (9) did find that immune serum was effective in treating monkeys suffering from meningitis experimentally produced.

Reports of patients treated with the Wollstein serum have been made by Torrey (10), Packard (11), and Dunn (12). Torrey and Packard each treated one case, and both, children 11 years old, recovered. Dunn treated eleven cases and all died. He considered that in none of these was the serum treatment started early enough to expect the best results.

In 1921, Neal (13) reported that five patients had been treated with anti-influenzal serum by the Meningitis Division of the New York Department of Health. One patient who was treated with a number of intraspinal injections of vaccine and a few injections of antiserum recovered. In 1933, Neal (14) stated that among 90 cases of influenzal meningitis under their care, there had been three recoveries. These recoveries she did not definitely ascribe to the use of influenza antiserum, but she mentions that she was impressed with the marked, though temporary, improvement in clinical symptoms which followed the use of the serum.

Notwithstanding the earlier failures and the fact that the anatomical conditions in influenzal meningitis are such as to render the local application of any specific treatment difficult, if not impossible, it was

[1] Certain of these strains were received from Dr. Martha Wollstein, Babies Hospital, New York, Dr. Ann G. Kuttner, Pediatric Clinic of Johns Hopkins Hospital, and the physicians listed in footnote 2, to all of whom the author is greatly indebted.

decided that in the light of the newer knowledge concerning these organisms, a further study of this problem should be made.

Production of Immune Serum in a Horse

In February, 1931, the immunization of a horse with Type b organisms was started.

The antigen used for the earlier as well as the later injections has been prepared by growing Type b meningitis strains for 18 hours on Levinthal agar (1) made with horse blood, and washing the bacteria from each plate with 6 cc. of 0.4 per cent formalin in 0.85 per cent NaCl solution. A fresh lot of vaccine has been prepared for each series of inoculations. Up to the present, twenty-seven different strains have been used. For the first vaccine, five strains were employed; then, as new strains were obtained, the old strains were discarded and the new ones substituted. Great precautions have been taken to employ only pure S cultures in the preparation of the vaccine.

The horse has been immunized by giving a series of five daily intravenous injections of the vaccine followed by a rest period of 9 days before beginning a new series. As marked reactions occurred following the injections, very small inocula of vaccine had to be employed. For the first series, two inoculations of 0.05 cc. of vaccine were made, then the amount was slightly increased for each of the remaining three injections of this series. For the first inoculation of each of the following series of injections, the amount given was a trifle greater than that of the first inoculum of the preceding series, and the dosage was then increased daily. This method of increase was continued until a large bleeding was made, after which the horse was allowed to rest for several weeks. In renewing the process of immunization following the rest, very small inocula were again at first employed, but the size of the dose was increased more rapidly than before.

After each injection, the horse has had a febrile reaction which has reached its maximum in 4 to 7 hours. This reaction has usually been greatest on the 1st and 2nd days of each series of injections. Besides the febrile reaction, the horse has at times had very labored breathing, increased heart rate, weakness of the legs, and marked injection of the blood vessels of the sclerae.

In order to follow the progress of the immunization, a small amount of blood was withdrawn from the horse before the first inoculation of each series, and the serum was tested for its content in type-specific antibodies by means of precipitation and agglutination reactions. For $3\frac{1}{2}$ months, a gradual increase in the precipitating and agglutinating power of the serum occurred. Since then, there has been no apparent change. Precipitation occurs when the Type b purified

capsular polysaccharide in dilutions up to one part in one million is added to the serum. Type b bacilli are agglutinated with disc formation in dilutions of the serum up to 1–80, and with granular clumping in higher dilutions up to 1–320.

The serological reactions are carried out at 37°C. for 2 hours, and the tubes are then kept in the ice box overnight before the final readings are made. If the tests are made at temperatures higher than 37°C., disc formation becomes less striking and agglutination becomes largely of the granular type.

The precipitation reaction appears to be the more specific test, and after 2 years of immunization the serum contains no precipitating antibodies for soluble specific substances derived from influenza bacilli other than Type b. On the other hand, after the horse had been under immunization for about a year, it was found that the serum had acquired the ability to agglutinate influenza bacilli of other types. This non-type-specific agglutination is probably due to the presence in the serum of an antibody against some fraction of the cell other than the soluble specific substance. Preliminary experiments indicate that this fraction is carbohydrate in composition, that it is present to some degree in all influenza bacilli, and that it is probably analogous to the C substance of Streptococcus (15) and Pneumococcus (16).

It has thus been possible to produce in a horse an immune serum which is highly specific for Type b influenza bacilli, as shown by precipitation tests. It now seemed important to determine, if possible, whether the serum would exert specific effects in the infected animal as well as in the test-tube. In case the serum were found to have therapeutic value, it also seemed important to learn whether quantitative differences in the relative value of the several lots of serum could be demonstrated.

Consequently, a series of experiments has been made to determine the action of the serum in infected animals.

The Effect of Immune Serum on Mice Infected with Hemophilus influenzae

Since the susceptibility to infection with *H. influenzae* varies markedly in individual mice, and since large inocula are necessary to produce lethal results, it seemed probable that a method of testing based on protective power for mice would not be suitable for determin-

ing the immunological value of this serum. Attempts were made, therefore, to evaluate the action of the serum in preventing or inhibiting invasion of the blood following intraperitoneal infections. The bacteria and serum were injected simultaneously, and at various intervals cultures were made of blood from the end of the tail, and the number of bacteria in the blood determined. In a few of the experiments, cultures were also made at the same time from the peritoneal cavity. The results of one of these experiments are given in Table I.

TABLE I

Protection of Mice against Hemophilus influenzae

Culture inoculum	Antiserum Lot 2	Blood cultures—hrs. after inoculation								
		Peripheral								Heart
		1	2	3	4	5	6	7	24	48
cc.	cc.									
0.5		++	+++	++++	++++	D				
0.5		++	+++	++	++++	++	+	+	+++	−
1.0		+++	++++	++++	++++	D				
1.0		++++	++++	++++	++++	D				
0.5	0.2	−	−	−	(2)	−	(1)	−	−	−
0.5	0.2	−	−	−	−	−	−	−	−	−
1.0	0.2	−	(2)	(5)	+	++	++	++	(1)	D 31 hrs. (2)
1.0	0.2	(2)	(1)	(6)	(5)	(6)	(2)	−	+	−

−, +, ++, +++, ++++ = none, few, moderate number, many, very many colonies that grew from 1 loopful of blood on Levinthal agar.

Numerals within parentheses indicate the actual number of colonies which grew from 1 loopful of blood.

D = death of animal.

The surviving mice were killed after 48 hours.

It is seen that in the serum-treated mice the invasion of the circulating blood by the bacteria was either prevented or limited, and that in those mice in which invasion occurred the severity of the septicemia was slight, and that three out of four mice survived. The fourth mouse lived for 31 hours, at no time did it have a severe septicemia, and from the heart's blood culture made at autopsy only two colonies developed. In other experiments, however, a few of the treated mice

have died as rapidly as the untreated ones. Yet in all experiments the serum-treated mice which succumbed have never had more than a mild septicemia, and in some instances the blood cultures have been sterile. Moreover, in the treated mice which have died the number of bacteria in the peritoneal cavity has been markedly reduced, and the bacteria have been swollen and globoid, and undergoing phagocytosis.

On the other hand, in the experiment recorded in Table I, within 1 hour the untreated mice had moderate to severe septicemia, and at the end of 5 hours three of the four mice were dead. The remaining mouse had a marked septicemia at the end of 24 hours, but the heart's blood culture was sterile when the mouse was killed at the end of 48 hours. In all of the experiments, blood cultures from the untreated mice which succumbed were positive, and cultures made from the peritoneal fluid of these animals showed heavy growths.

All of the animals, treated as well as untreated, have exhibited certain toxic symptoms such as diarrhea and conjunctivitis, yet these symptoms have disappeared more rapidly in the treated animals than in the untreated animals which recovered.

Control experiments have shown that the action of the horse serum is type-specific, since no effect of the serum could be demonstrated in mice infected with Type a or R strains. Moreover, it has been shown that the administration of normal horse serum has no effect on mice infected with Type b strains.

While it was possible to show that the immune horse serum has a specific effect on mice infected with Type b influenza bacilli, it was difficult to estimate this effect quantitatively. Consequently, it was decided to attempt to determine the effect of the serum on larger animals, and since the rabbit, in proportion to its weight, is apparently the least resistant of the ordinary laboratory animals to infection with *H. influenzae*, this animal was chosen for the experiments.

The Effect of Immune Serum on Rabbits Inoculated Intravenously with Hemophilus influenzae

Rabbits were inoculated intravenously with given amounts of culture, and after 30 minutes to an hour blood cultures were made on plates. Definite amounts of immune serum were then injected intravenously. Shortly after the serum treatment, and then at hourly

intervals, blood cultures were made. Febrile and other symptomatic reactions were also observed at hourly intervals.

The culture, Strain 306S, employed in these experiments, was originally obtained from the spinal fluid of a patient suffering from meningitis. It was passed through a number of rabbits, and its virulence for rabbits at the time these experiments were made was such that 0.5 cc. to 0.25 cc., and at times even 0.1 cc. of a broth culture, killed.

The rabbits employed were approximately 1500 gm. in weight, and those used in a single experiment were of the same litter, or they were of the same breed and nearly of the same age.

It was observed that 30 minutes to an hour after infection the rabbits had a high grade septicemia, but that 5 minutes after the administration of immune serum the number of bacteria in the blood was reduced, and that after an hour the blood contained few organisms, or was sterile. Most of the serum-treated rabbits survived, but even in the ones that died the reduction in the number of bacteria in the blood was noteworthy. In the control rabbits, which received no immune serum, the degree of septicemia increased, and rarely did a rabbit recover. The protocol of one of these experiments is given in Table II.

In this experiment, each of six rabbits was inoculated with 0.5 cc. of a broth culture. 30 minutes later cultures were made on plates with a loopful of blood from the ear vein, and then three of the rabbits were treated intravenously with immune serum, two with normal serum, and one was left untreated. A seventh uninfected rabbit received serum only. After another interval of 30 minutes, and then at hourly intervals, the blood cultures were repeated.

As shown in the table, 30 minutes after infection all the rabbits had a massive septicemia. But in the rabbits treated with immune serum this bacteremia was so quickly reduced that 30 minutes after treatment only a few or no colonies developed on the plates, and, except in one instance, all subsequent cultures were sterile. In this exceptional case, the rabbit receiving the smallest amount of serum, six colonies developed in the culture obtained 24 hours after treatment. Despite the fact that in all the serum-treated animals the blood was sterilized, one of the animals was found dead at the end of 24 hours.

In contrast to the rapid disappearance of the bacteria in the serum-

TABLE II

Protection of Rabbits against Hemophilus influenzae

Rabbit No.	Culture	Serum	Blood cultures—hrs. after inoculation of culture										
			½		1	2	3	4	5	6	7	24	48
	cc.	*cc.*											
1	0.5	Immune* 0.5	+++	Inoculation of serum	(5)	–	–	–	–	–	–	(6)	–
2	0.5	1.0	+++		+	–	–	–	–	–	–	D–	
3	0.5	2.0	+++		–	–	–	–	–	–	–	–	–
4	0.5	Normal 1.0	+++		+++	+++	++++	++++	D++++				
5	0.5	2.0	+++		+++	+++	++++	++++	++++	++++	++++	++++	+
6	0.5	–	+++		+++	+++	++++	++++	++++	++++	++++	D++++	
7	–	Immune 2.0											

–, +, ++, +++, ++++ = none, few, moderate number, many, very many colonies which grew from 1 loopful of blood.

Numerals within parentheses indicate the actual number of colonies which grew from 1 loopful of blood.

D = death.

* Immune serum from Lot 5.

treated rabbits, the degree of bacteremia increased in the rabbits which were not treated, or which were treated with normal serum.

One of these rabbits died at the end of 5 hours, and another within 24 hours. Both had many organisms in their blood at the time of death. The third rabbit had a massive septicemia at the end of 24 hours, but at the end of 48 hours only a few organisms grew from the blood. In this animal the blood cultures continued positive for several days. It then developed another infection and was killed. The rabbit which served as the serum control showed no abnormal reactions.

A number of experiments similar in plan to the one just described were made, and the results were similar. Furthermore, similar results were observed when the serum was administered before, or simultaneously with, the culture. In one experiment, however, in which the dosage of culture employed was larger, all the rabbits died. Even under these circumstances, however, there occurred a marked reduction in the number of bacteria in the blood of the animals treated with immune serum, as compared with the number of bacteria in the blood of the control animals. It has been found, as the immunization of the horse has progressed, that the amounts of serum necessary to bring about sterilization of the blood have become smaller.

The immune horse serum, therefore, possesses the power of sterilizing the blood of rabbits infected with homologous organisms, unless the initial inoculum of culture is so great that the animal is completely overcome by the toxicity of the injected bacteria. On the other hand, the serum apparently does not prevent the toxic symptoms which follow the inoculation of animals with *H. influenzae*, since these symptoms occur in the treated as well as in the untreated animals.

These symptoms, which have been described by others, are rapid and labored breathing, loss of muscle tonus, increased peristalsis, refusal to eat, and increased secretion from the conjunctivae. Another feature, apparently not previously mentioned, is injection, and at times hemorrhages, of the scleral vessels. This reaction begins about 1 hour after inoculation, and usually persists for about 24 hours. It apparently corresponds to the injection of the blood vessels and hemorrhages in the internal organs which are seen at autopsy in rabbits after inoculations of *H. influenzae*. A further toxic manifestation which occurs shortly after infection is a change of temperature, which may be either lowered or elevated. In the rabbits which survive, there is a rapid elevation of the temperature, which usually returns to normal in less than 48 hours, and the latter occurs even though the bacteremia may still be present.

Although the administration of serum does not prevent the occurrence of these toxic symptoms, it apparently does shorten their duration. This is probably associated with the inhibition of growth of the bacteria by the serum.

The Action of Immune Serum in Preventing the Lesions Induced by Intracutaneous Injections of Hemophilus influenzae

The first description of skin lesions induced by the injection of Pfeiffer bacilli was made in 1894 by Kruse (17). He inoculated large doses of bacteria subcutaneously into rabbits. The resulting localized lesions were edematous and hyperemic, yet cultures made from them after 24 hours were sterile. After a few days the inflammation subsided, but a hard mass, potato-like in consistency, persisted for a number of days.

In a previous paper (1), the author has described the lesions resulting from the intracutaneous injection of cultures of S and R influenza bacilli in rabbits, and has drawn attention to the employment of this technique for determining the relative virulence of different strains.

Although previous observations had been made of the skin lesions induced by *H. influenzae*, it seemed important, before studying the action of the immune serum on the development of these lesions, to make a more detailed study of the effects produced by the injection of different amounts of various strains, both living and dead.

It was found that when a massive inoculation, consisting of the living S organisms concentrated from 1 cc. of a broth culture, is given intradermally, the localized lesion may reach 20 to 40 mm. or more in diameter. The lesion is markedly edematous, at first bright red, then purple-red in color. Later, the center becomes necrotic and a scab forms which covers a thick fibrinopurulent exudate—rarely is there any discharge. The reaction usually begins to decrease after 48 hours, but some inflammatory reaction remains for 5 to 7 days. As the inflammation subsides, a large hard palpable area of induration becomes evident. This may persist for as long as 20 days.

When massive inocula of R organisms are injected, lesions of almost equal severity develop. Furthermore, similar reactions develop if large amounts of dead organisms, either S or R, are given. However, the intensity of the lesions induced by the different forms bears a very definite relationship to the amount of culture injected. When small amounts are given, it is observed that the living S forms have a much greater capacity to induce lesions than do the dead S, or living or dead R forms. Of the three latter forms, there seems to be no significant difference in the reactions which they induce.

To illustrate the capacity of these forms of bacteria to induce lesions, Table III is given, in which is indicated the relative severity of the reactions usually observed.

Since dead forms of these bacteria are capable of inducing lesions, it is obvious that at least some of the effects of intracutaneous inoculations are due to preformed substances present in the bacterial cells. Further, since the lesions induced by living R forms are no more severe than those induced by the dead R forms, it seems probable that in the case of the R forms the lesions are entirely due to preformed substances, the living R forms being quickly killed after the inoculation.

TABLE III

Comparison of Skin Lesions Induced by S and R Forms of Hemophilus influenzae

Amount of culture in 0.2 cc. volume	Qualitative differences in the lesions induced by		
	Living S	Living R	Dead S or R
cc.			
1.0	++++	+++	+++
0.1	+++	+	+
0.01	++±	±	±
0.001	++	−	−
0.0001	+	−	−
0.00001	±	−	−

−, ±, +, ++, +++, ++++ = no reaction, very mild, mild, moderate, severe, and very severe reaction.

In the case of the S forms, however, it is noted that a much smaller dosage of living than of dead bacteria will induce an evident lesion, and in this instance it seems that living S forms are able to multiply and thus produce sufficient irritating substance to give rise to reactions.

Preliminary experiments showed that if a certain amount of immune serum were added to massive doses of the culture, some effect on the extent of the lesion was observable, but that if smaller doses of culture were employed the inhibiting effect of the immune serum was much greater, and that with still smaller doses the production of a lesion might be completely prevented. It was found that the actual amount of serum injected made little difference in the severity of the lesions; if the dose of culture was not too great, small amounts of serum within

certain limits were as effective as large amounts. Furthermore, it was observed that when the dose of culture was so large as to produce lesions in spite of the admixture with serum, the lesions were very similar in size to those induced by like numbers of heat-killed bacteria given alone. Hence, it appeared that the serum could only inhibit the action of a definite number of bacteria, and that the action of the serum consisted in preventing the growth of the bacteria rather than in neutralizing the toxic or irritating substances.

It was therefore evident that in employing skin inoculations to make quantitative tests of the inhibitory action of different lots of serum, it would be necessary to determine with considerable care the optimal dosage of culture to be employed in the tests. A series of observations, therefore, was made, employing various doses of culture alone, and also the same amounts of culture mixed with varying amounts of immune serum.

The culture employed in the tests was Strain 225S, isolated from a patient suffering from meningitis. The strain was kept under optimal conditions to prevent the development of R variants. The technique employed in cultivating and studying the characteristics of the culture was the same as that described previously (1). For the tests, 24 hour broth cultures were employed, and physiological salt solution was used as a diluent rather than broth, as the whole broth alone sometimes causes slight reactions. To prevent injury to the bacterial cells taking place in the salt solution, the injections were made as quickly as possible after diluting the culture. In all cases, 0.1 cc. of the culture or culture dilution was mixed with 0.1 cc. of salt solution, or with 0.1 cc. of whole or diluted serum, the amount of fluid injected in all cases being 0.2 cc.

On the day preceding the tests, the hair was removed from the flanks of the rabbits with electric clippers. Gray rabbits with thick white skins have been found most suitable for the tests.

It was found that when the largest inoculum of culture (0.1 cc.) was injected alone, there was induced a hyperemic edematous lesion 20 to 30 mm. in diameter, with a small central area of necrosis. This inflammatory reaction lasted for 4 to 6 days, and one large palpable area of induration, or several smaller areas, persisted for about 10 days longer. Smaller amounts of the culture induced lesions of less severity and with less induration, but the lesions did not differ markedly in surface area unless the inocula were less than 0.0001 cc. of culture. Amounts less than 0.0001 cc. sometimes induced lesions as large as 15

mm. in diameter, but these rapidly diminished and rarely exceeded 10 mm. at the end of 72 hours, and no palpable areas of induration persisted.

When the serum was injected together with the culture, it was found that 0.001 cc. of culture was the largest dose that could be completely neutralized by the addition of the immune serum, no matter how large an amount of serum was employed. In other words, if doses larger than 0.001 cc. of culture are used, there is apparently sufficient preformed substance present to induce lesions, even though the growth of the bacteria may be completely inhibited. It therefore became obvious that 0.001 cc. was the largest dose of culture which could be used for carrying out a series of tests with different sera. And since this amount was at least ten times greater than an amount (0.0001 cc.) which could produce a persistent lesion, it was decided that 0.001 cc. of culture would be the most satisfactory dose to employ. It was arbitrarily decided that a reaction was to be considered negative if at no time it exceeded 10 mm. in diameter and had completely disappeared within 72 hours.

When the horse was bled at the different intervals, tests were made with various dilutions of the serum, employing 0.001 cc. as a constant standard dose of culture. Control tests were also made with mixtures of culture and normal serum, and with the culture alone. The results of three of these tests are given in Table IV.

The lesions are described by means of linear measurements, but this method of expressing the difference in the lesions is inadequate, as it does not indicate differences in degree of hyperemia and edema.

It will be seen that whereas the administration of normal serum had no effect in reducing the size of the lesions resulting from the intradermal injection of 0.001 cc. of the standard culture, the addition of immune serum had a marked effect on the lesions. Moreover, distinct differences in the effects produced by sera from various bleedings were evident Employing the standards mentioned above, it is seen that while 1/200 cc. of serum of Lot 1 was necessary to render the skin reaction negative, only 1/600 cc. of serum of Lot 2 was required, and 1/800 cc., or possibly less, of Lot 3.

It is obvious, therefore, that by means of this technique the protective action of immune serum may be demonstrated, and, moreover,

that quantitative differences in the relative protective action of different lots of serum may be detected. Lot 1 serum was obtained 4 months after the immunization of the horse was undertaken, Lot 2 serum 3 months later, and Lot 3, 3 months after Lot 2. The results indicate, therefore, that with the continued immunization there occurred a progressive increase in the power of the serum to prevent

TABLE IV

Influence of Immune Serum in Preventing Lesions in the Skin of Rabbits

Rabbit No.	Readings	Serum	Area of lesion induced by									
			Serum plus 0.001 cc. of culture							Culture alone		
			1/10 cc.	1/100 cc.	1/200 cc.	1/400 cc.	1/600 cc.	1/800 cc.	1/1000 cc.	0.001 cc.	0.0001 cc.	0.00001 cc.
	hrs.		*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
1	24	Immune		10x10	4x7	14x11	13x12			25x22	18x16	
	72	Lot 1		—	—	10x10	12x12			22x21	10x10	
2	24	Lot 2		8x8	9x9	7x7	8x8	10x12		18x16	10x11	
	72			—	—	—	—	8x10		19x17	10x10	
3	24	Lot 3	—	3x3	—	—	2x2	7x7	10x11	*	15x15	12x12
	72		—	—	—	—	—	—	—		11x12	7x8
	24	Normal	20x15	15x20	15x13							
	72		20x15	15x15	13x11							

Total volume of each inoculum = 0.2 cc.

The sera alone caused no reactions.

Protective titre of immune serum: Lot 1 = 1–200; Lot 2 = 1–600; Lot 3 = 1–800+.

* Material was lost.

the occurrence of skin lesions following the injection of a definite number of Type b influenza bacilli.

The immune serum was also tested with other Type b strains, and the results were the same as when the standard Strain 225S was employed. That the action of the serum was type-specific was demonstrated by observing the lesions induced by the injection of mixtures of serum and living and heat-killed organisms of the other types and R

forms. Under these conditions, no preventive action of the serum could be detected. However, the serum used in these cross-protection tests was from the second bleeding, which was made before the appearance of the antibody which seems to be similar to the C antibody, and it is not known what influence this antibody might have on heterologous type skin infections.

The Action of Immune Serum in Patients Suffering from Meningitis

The experimental studies have indicated that the serum of a horse immunized against Type b *H. influenzae* exerts a specific action on these organisms, not only in the test-tube, but also in infected animals. It was decided to study the therapeutic action of this serum in a small series of patients suffering from meningitis due to *H. influenzae*, Type b. As cases of this disease occur mostly in young children and have not been available in this hospital, the cooperation of certain physicians likely to meet with cases of this disease has been enlisted. The writer desires here to express thanks to the physicians who have kindly supplied the histories of the patients.[2]

Eighteen patients have now been treated by means of intrathecal injections of immune horse serum supplied by us. Owing to difficulties in making prompt diagnosis of the bacteria concerned, and lack of opportunities for careful study of the cultures, certain of the cases cannot justifiably be included in discussing the action of the serum. In three cases, the influenza bacilli isolated proved to be not of the Type b form. In one of these cases, the organisms were found to be of Type a, in another of Type f, and in the third case the organisms which grew from the spinal fluid obtained on four occasions early, as well as late in the disease, produced only R colonies. Inasmuch as the serum is considered to be type-specific, no action was expected in patients

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suffering from infections due to heterologous organisms. Nevertheless, in one of these cases, following treatment with the immune serum, the patient apparently recovered from an infection with *H. influenzae* of a heterologous type.

This case, W. B., treated at the Jersey City Hospital, was not typically one of primary meningitis in a child, but was a case of secondary meningitis following traumatic fracture of the skull and laceration of the face occurring in a man 51 years of age. 6 days after the injury he developed signs of meningitis, and 4 days later cultures from the cloudy spinal fluid revealed the presence of *H. influenzae* Type a. The physician was anxious to employ treatment with immune serum, and intraspinal injections of 20 cc. each were made once or twice daily over a period of 19 days. 7 days after the treatment was begun, cultures from the spinal fluid became sterile and remained so. During the period of treatment marked improvement in the patient's physical and mental condition occurred, but about 3 weeks after the treatment was stopped his condition again became worse and he died 1 week later. At autopsy, there was no evidence of meningitis, but a brain abscess was found, cultures from which showed the presence of streptococci and staphylococci, but no influenza bacilli.

Whether or not the serum had any effect in this case is uncertain. Influenza bacillus meningitis rarely occurs in adults, and it is less frequently fatal than in children. Its occurrence in adults has been reviewed by Wollstein (18), Rivers (19), Bloom (20), and others.

The other two cases of this group, the one in which Type f organisms were found, and the one in which the organisms were all of the R form, ended fatally, and there was little evidence that the administration of the serum had any effect on the course of the disease.

There were two further cases in which a thorough study was not made of the infecting organisms. The determination of type was made solely on the agglutination reaction in the immune horse serum, and, in the light of further experience, the conclusion that the organisms were of Type b must be considered doubtful.

There remain thirteen cases in which the infecting organisms were shown conclusively to be of Type b. Unfortunately these patients were treated under widely varying conditions, and by different methods. The ages of these patients ranged between 2 months and 7 years, nine of them being 3 years, or younger. Among these thirteen cases, there was only one in which recovery occurred.

Case I.—This patient, M. P., 2 years of age, was admitted to the Jewish Hospital of Brooklyn on Apr. 24, 1933. There had been a discharge from the ear for 4 weeks. For 3 days the child had had signs of muscular incoordination, as he was reported to have fallen several times. The night before admission he vomited and had a chill, which was followed by a rise in temperature. The following morning he seemed well, but in the evening his temperature again rose and he appeared very limp, drowsy, and listless. The next day he was admitted to the hospital. A diagnosis of meningitis was made, and he was treated with meningococcus antiserum. On the 2nd day, the organisms which grew in the culture from the spinal fluid, and also in the culture from the blood, were identified as influenza bacilli, and treatment with specific immune serum was started. On this day he was given two intravenous and two intralumbar injections of immune serum, all of 20 cc. each. On each of the 4 succeeding days he was given two intralumbar treatments of 20 cc. of immune serum to which 2 cc. of fresh human serum had been added, on each of the next 2 days one treatment of 10 cc. of immune serum plus 2 cc. of fresh serum, and on the following day one intralumbar injection of 10 cc. of immune serum alone. On the 2nd day of specific treatment he was also given an intravenous injection of 10 cc. of immune serum. After the first 24 hours of treatment, cultures from the spinal fluid were sterile. This was after two positive cultures had been obtained from the spinal fluid, and before any fresh serum had been injected. The blood culture was positive on admission, and no further cultures were made from the blood until 1 week later, at which time no growth occurred. During the period of specific treatment, the number of leucocytes in the spinal fluid gradually decreased from 8900 to 460 per c.mm., and 10 days later the cell count was 5. Clinically, the child progressively improved, the meningitic symptoms gradually disappearing. The temperature slowly dropped, and after May 2 remained normal. The patient, apparently well, was discharged May 13.

Shortly after admission to the hospital, nose and throat cultures were made from which Type b influenza bacilli were grown. 10 days after discharge, cultures were again made from the nose and throat, and from the nose culture Type b organisms were again recovered. The latest cultures were made on June 29 from excised tonsils and adenoids, and on the plates a few colonies of Type b organisms and many colonies of R forms developed.

In two of the thirteen cases, the spinal fluid became sterile following the administration of immune serum, and remained so for periods of 7 to 14 days, but in both instances the organisms reappeared and death ensued. Brief reports of these cases follow.

Case II.—R. S., 2½ years old, was admitted to the Babies Hospital, New York City, Mar. 8, 1932. A diagnosis of influenzal meningitis was made, and the child was treated intraspinally with immune serum to which fresh human serum was

added. For 4 days the number of influenza bacilli in the spinal fluid diminished, on the 5th day no growth was obtained, and for the next 6 days the cultures were sterile. During this time the serum treatment was continued and the child improved clinically. However, the child then developed otitis media, streptococci were recovered from the discharge, and influenza bacilli again appeared in cultures from the spinal fluid. After this, cultures from the spinal fluid were again sterile for several days, but the influenza bacilli reappeared once more and were present in all subsequent cultures until death, which occurred on Apr. 9.

Case III.—J. B., 3½ years old, was admitted to Beth-El Hospital, Brooklyn, Dec. 5, 1932. The child had been ill for 4 days, and for 3 days meningitic symptoms had been present. On admission, a lumbar puncture was made, a cloudy fluid was withdrawn, and the child was treated with meningococcus antiserum. But, on examination of a smear of the spinal fluid, a diagnosis of influenzal meningitis was made, and later that day the child was treated with specific immune serum. For the next 7 days the child was given two daily intralumbar injections of serum, of approximately 20 cc. each. Positive cultures of *H. influenzae* were obtained from the spinal fluid daily for 4 days after the beginning of treatment, then the cultures became negative (Dec. 10) and remained so until Dec. 24. On Dec. 12 the spinal fluid was clear, the cell count 100, and the sugar content normal, but as the temperature continued elevated a block was suspected. A cisternal puncture was made and fluid of the same appearance as that obtained from the lumbar tap was withdrawn. This fluid was replaced by serum. Cultures made from this fluid were sterile. From Dec. 12 to 22 the child was given one daily intralumbar injection of immune serum, 20 cc. on each occasion. During this time the child showed improvement and ate and slept well. The strabismus which had been present disappeared, but double otitis media developed and both drums were punctured on Dec. 18. On Dec. 24 the child's condition became much worse. Bilateral mastoiditis developed, and it was found that the spinal fluid had again become positive for influenza bacilli. 4 days later both mastoids were opened. The serum treatment was continued after the operation, but the child progressively grew worse and died Jan. 1, 1933. No fresh serum was used in the treatment of this case.

In another case, there was a reduction in the number of bacteria in the spinal fluid following intraspinal treatment, and on one occasion no organisms were recovered either from slant or broth cultures.

Case IV.—R. F., 5 years of age. The onset of this child's illness was very sudden, and she was admitted to the New Haven Hospital Nov. 25, 1931, on the 1st day of illness. A diagnosis of meningitis was made, and she was given an immediate intraspinal treatment with meningococcus antiserum. An examination of a smear of the spinal fluid, however, showed the presence of Gram-negative bacilli, and treatment with *H. influenzae* antiserum was begun. The next day the

child was given two intraspinal treatments, the 5 succeeding days one intraspinal or intracisternal treatment daily, and the 8th day two intraspinal treatments. No definite clinical improvement was noted, however, and the serum treatment was discontinued. The child died on the 26th day. On admission, the blood culture was positive, but on the 3rd day after treatment, and also on three subsequent occasions, it was negative, the last negative culture being obtained 10 days after the cessation of serum treatment. However, 4 days later a positive blood culture was again obtained, and death occurred the 4th day following. The spinal fluid cultures following the administration of the serum showed a reduction in the number of bacteria present, and on one occasion, after three intraspinal treatments, the cultures were sterile. But on all subsequent occasions the spinal fluid cultures were positive.

In each of three other cases (Cases V, VI, and VII) in which treatment was commenced on the 7th, 5th, and 2nd days of illness, respectively, there occurred a temporary decrease in the number of bacteria in the spinal fluid following the treatment with immune serum. Later, the organisms again became numerous in the spinal fluid and remained so until death. In Cases V and VII, the cultures from the blood, which were positive before treatment was commenced, later became sterile.

In Case V, two positive blood cultures were obtained on 2 successive days. 2 hours after the patient had received 20 cc. of immune serum intraspinally, and 20 cc. intravenously, the cultures from the blood were sterile. Blood cultures were also sterile 2 days later, but on the following day, 24 hours after serum treatment had been discontinued, the blood cultures were again positive. The patient died 2 days later.

In Case VII, 2 days after a total amount of 60 cc. of influenzal antiserum plus 15 cc. of fresh human serum had been given intraspinally, and 120 cc. of influenzal antiserum intravenously, the blood culture was negative. Blood cultures on the following day were also negative. The child lived 2 days longer, during which time the specific treatment was continued, but no reports of further blood cultures were made.

Among the remaining six cases, there occurred no significant changes in the condition of the patients or in the character of the spinal fluid following the administration of immune serum. In two of these only intracisternal treatments were given on account of inability to withdraw fluid by the lumbar route, and in one, two intraspinal injections were made, after which the serum treatment was discontinued because of inability to withdraw spinal fluid by this route.

It is realized that the results obtained in the treatment of these thirteen cases do not indicate that this form of specific therapy, carried out under the given conditions, was of great practical value. It must be borne in mind, however, that influenzal meningitis is a very serious condition, and that without specific treatment almost all of those afflicted die. Moreover among the cases here reported two were complicated by pneumonia and empyema. Six were treated very late in the course of the meningitis, and in certain instances treatment was carried out only over short periods of time. It is possible that in a group of cases treated earlier in the course of the disease with greater intensity, and over prolonged periods, the results might be better.

The most important evidence presented by this study, indicating that the administration of the serum may exert an influence on the course of the disease, is given by the results of cultures from the blood and spinal fluid before and after the administration of serum. In seven of the cases, influenza bacilli grew in cultures from the blood before specific serum treatment was administered. In two of these cases no further reports were obtained on the course of the blood infection. In one case, the patient was suffering also from pneumonia, and the septicemia was uninfluenced by the administration of serum. In the remaining four cases with positive blood cultures, the blood became sterile after treatment with serum; in one instance (Case I) the patient recovered, in another (Case VII) there was no report of a recurrence of blood invasion, while in two cases (Cases IV and V), in which treatment was discontinued, the blood cultures again became positive before death. The total number of cases is small, but it seems not unlikely that, in the four cases mentioned, the administration of immune ser m had the effect of at least temporarily sterilizing the blood.

The results of the intrathecal injection of immune serum on the bacteria in the spinal fluid varied, but in certain instances, at least, the serum seemed to have a definite effect. In one case (Case I) the bacteria disappeared from the spinal fluid following treatment, and recovery occurred. In two cases (Cases II and III) the spinal fluid became sterile and remained sterile for 7 and 14 days, respectively. In one case (Case IV) there occurred a reduction in the number of bacteria, and on one occasion the culture was sterile. In three other cases (Cases V, VI, and VII) there occurred a reduction in the number of bacteria, as shown by smears and cultures. In the remaining six

cases, no changes in the number of bacteria in the spinal fluid were noted.

Recently, Ward and Fothergill (6), and Ward and Wright (5), have reported concerning the treatment of eight cases of influenzal meningitis with an immune serum produced by immunization of a horse with meningitic strains of *H. influenzae.* In one case recovery occurred, and in five, after treatment, the cultures of the spinal fluid were sterile for periods of from 1 to 14 days. In all these five cases the bacteria later appeared in the cultures and death ensued. In the treatment of these cases fresh human serum was added to the immune serum. The previous experimental studies of these writers had led them to the conclusion that the action of *H. influenzae* anti-serum is bactericidal, brought about by the action of antibody and complement upon the antigen. As they had been unable to demonstrate the presence of complement in the spinal fluid of patients suffering from influenzal menigitis, they considered that complement should be added to the immune serum before making intrathecal injections.

In the treatment of the patients reported in the present paper, in certain cases small amounts of fresh serum were mixed with the immune serum, and in other cases the immune serum was given alone. Our data do not permit definite conclusions to be drawn concerning the importance of the addition of fresh serum. In the one case (Case I) which recovered, however, the spinal fluid cultures became sterile after the injection of the immune serum without the addition of fresh serum, and in another instance (Case III) the spinal fluid cultures became sterile and remained so for a period of 14 days following the administration of immune serum alone. In Case IV, in which a reduction in the number of organisms occurred, and in which the fluid was sterile on one occasion, no fresh serum was added to the immune serum. In three other cases in which there resulted temporary sterilization of the spinal fluid or a reduction in the number of bacteria present, fresh serum was added to the immune serum.

SUMMARY

In this communication, further evidence has been given which supports the view that the majority of the strains of *Hemophilus influen-*

zae giving rise to meningitis are of the same serological type. Forty strains have now been examined, and thirty-seven have been of Type b.

A horse has been artificially immunized with Type b strains isolated from the spinal fluid of patients. By precipitation tests with the capsular carbohydrate, the serum has been shown to be highly type-specific. For the first $3\frac{1}{2}$ months of immunization, the *type-specific* antibody content of the serum increased steadily. Later, in spite of continued immunization, there occurred no apparent increase.

By means of animal inoculations, it has been shown that the anti-serum has an anti-infectious action. If mice, inoculated intraperitoneally with Type b organisms, were also given serum, the bacteria did not invade the blood, or did so to only a limited degree. But the recovery of the treated mice was found to be inconstant. In rabbits infected intravenously and later treated by the same route, the number of bacteria in the blood stream was quickly reduced and sterilization followed. In the experiments it was necessary that the dosage of the culture be not too large, as influenza bacilli contain a substance which, artificially introduced into mice and rabbits, gives rise to marked toxic reactions. This substance is apparently not neutralized by the anti-serum. However, it was found that among the surviving animals, those treated with immune serum returned to the normal state more quickly than did the animals not so treated.

The anti-infectious action of the serum has further been demonstrated by a study of its effect on the lesions which follow inoculations of type-specific bacteria into the skin of rabbits. Again it was found that for any effect of the serum to be manifested it is necessary that the dosage of bacteria be limited, since if large numbers of bacteria are introduced into the skin the development of lesions cannot be completely inhibited, no matter how large doses of serum are employed. As the number of living S organisms which cannot be neutralized is roughly equivalent to the number of R or heat-killed bacteria which may produce a lesion, it seems that there is some preformed irritating substance in the bacterial cells which may give rise to lesions, even if the bacteria are killed or inhibited in their growth. In order to demonstrate the protective action of immune serum, therefore, it has been found necessary to employ a dosage of culture so small that if the bacteria are immediately killed, or their growth inhibited, no lesion

results. Employing immune serum under these conditions, it has been found that the ability of the serum to prevent the occurrence of skin lesions has progressively increased with continuing immunization of the horse.

A series of eighteen patients suffering from influenzal meningitis has been treated with Type b antiserum. Following the use of serum, recovery occurred in one patient of the series, and in two, although the patients ultimately died, the spinal fluid cultures became sterile and remained so for periods of 7 to 14 days. In four other cases, the spinal fluid cultures showed, temporarily, either no growth of bacteria, or a reduction of their number. Among five patients in whom septicemia was present before treatment, in four the blood cultures, after treatment with serum, became sterile.

The number of patients treated has been small, and the treatments were carried out under widely varying conditions. It is difficult, therefore, to draw conclusions regarding the actual value of this form of therapy, or the best methods of procedure. The clinical results, however, indicate, as do the experimental, that the serum has a definite anti-infectious action. The experience is too limited to permit final conclusions regarding the importance of the addition of fresh (complement-containing) serum to the immune serum. Further experience, under more accurately controlled conditions, may show that the serum has greater practical value in treatment than is shown by the mortality results in this series of cases.

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CHEMOIMMUNOLOGICAL STUDIES ON THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS

I. The Isolation and Properties of the Acetyl Polysaccharide of Pneumococcus Type I

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During the past several years it has become increasingly evident that Pneumococcus Type I possesses type-specific immunological properties not completely accounted for by those of the capsular polysaccharide in the form in which it was first chemically isolated and identified in this laboratory as the soluble specific substance (1–3). This fact was foreshadowed in the earlier studies of Perlzweig and his coworkers (4, 5), and has since been amply demonstrated by a number of European and American investigators, notably by Schiemann and his collaborators (6, 7) abroad, and by Enders (8, 9), Sabin (10), Wadsworth and Brown (11, 12), Ward (13) and Felton (14) in this country.

In view of this evidence, a further study has been made of the chemical and immunological properties of the soluble specific substance of Pneumococcus Type I, with the hope of acquiring a fuller knowledge of the nature of the relationship existing between the specifically reacting derivatives studied by other investigators and the type-specific polysaccharide previously described in this laboratory. The results of this study form the subject matter of the present report.

Evidence will be presented that the soluble specific substance has now been isolated in a chemical form more closely approximating that in which it exists as a natural constituent of the cell capsule. It will be shown that the type-specific carbohydrate present in the intact bacterial cells, and in filtrates of autolyzed broth cultures has been chemically identified as an acetyl polysaccharide. This naturally occurring acetyl polysaccharide differs chemically from the spe-

cific carbohydrate as originally isolated, principally in respect to the presence of acetyl groups which, as will be pointed out, endow the native substance with additional specific properties not possessed by the polysaccharide after removal of these labile groups by alkaline hydrolysis.

Owing to the marked instability of the acetyl groups and the ease with which they are removed by treatment with alkali, the soluble specific substance as originally isolated will be shown to represent the deacetylated polysaccharide which, although still retaining the dominant type specificity of the native substance, has through the loss of its acetyl groups suffered a corresponding loss of certain specific properties possessed only by the acetyl polysaccharide itself. Thus, specific differences between the properties of the cell fractions studied by other investigators and those of the soluble specific substance as originally defined, now appear to be due to the presence or absence of acetyl groups in the polysaccharide molecule. Indeed, so distinctive are the immunological reactions of the acetyl polysaccharide and those of its deacetylated derivative, that it is now possible to clarify many of the apparently conflicting views still current concerning the nature and properties of the specific carbohydrate of Pneumococcus Type I.

I. Isolation of the Acetyl Polysaccharide of Pneumococcus Type I

Methods for the isolation of the acetyl polysaccharide differ from those previously described for isolation of the soluble specific substance, principally in the avoidance of the use of an excess of alkali in the chemical procedures employed. The following methods have been used in the recovery and purification of the acetyl polysaccharide from the bacterial cells and concentrates of autolyzed cultures.

1. Bacterial Cells.—The bacterial cells from 18 hour broth cultures of Pneumococcus Type I, grown in 3 liter lots, were collected by centrifugation. The unheated cells from each lot were taken up in 50 cc. of sterile saline and stored in the ice box until the bacteria from 50 liters had been collected. The suspension of partially autolyzed cells was then treated with 15 cc. of N/1 acetic acid and heated in an Arnold sterilizer for 30 minutes. The precipitate of coagulated protein and bacterial debris was removed by centrifugation, and washed several times. The washings together with the original supernatant were concentrated to 150 cc. *in vacuo.* 150 cc. of alcohol were added to the concentrated liquid. The copious precipitate, containing the specific polysaccharide, was separated

by centrifugation, and the alcoholic supernatant liquid was discarded. The carbohydrate was dissolved in 75 cc. of water, 1 cc. of N/1 acetic acid was added and the small amount of insoluble precipitate which formed was separated and discarded. The polysaccharide was precipitated from the solution by the addition of an equal volume of alcohol, the precipitate was recovered and again dissolved in 50 cc. of water. The solution of polysaccharide at this point was clear and colorless. It gave none of the usual protein tests. The solution was acidified by the addition of 2 cc. of 5 N HCl and dialyzed until no chlorine ion was detectable in the dialysate. At this point, a small amount of degraded (deacetylated) polysaccharide which had separated from the solution was removed by centrifugation. The viscous, clear and colorless solution of the specific acetyl polysaccharide was precipitated by pouring it into 10 volumes of acidulated acetone. The polysaccharide was separated by filtration on a porous, sintered glass funnel, and was washed with absolute alcohol and ether. 0.5 gm. of dry substance was recovered.

2. *Autolyzed Broth Cultures.*—50 liters of 0.5 per cent dextrose broth were seeded with an actively growing culture of Pneumococcus Type I, and incubated at 37°C. for 5 days during which time the bacterial cells had undergone marked autolysis. The reaction of the culture fluid after growth and autolysis had occurred was distinctly acid. The 50 liters of autolyzed broth culture were autoclaved and concentrated to one-tenth of the original volume in a steam kettle. The concentrated material was cooled to 0° and neutralized, while stirring, with solid sodium bicarbonate, 6 liters of alcohol were added to the concentrate and after standing for 18 hours at room temperature, the clear, dark supernatant liquid was syphoned off. After removal of the excess fluid by centrifugation, the precipitate was dissolved as completely as possible in 800 cc. of water. The deep brown and turbid aqueous solution containing the pneumococcus polysaccharide was clarified by centrifugation at high speed. The precipitated bacterial debris and coagulated protein was next washed with slightly acidulated water, until the washings gave only a faint Molisch test. Four to five washings were found necessary. To the combined solution of polysaccharide and wash waters, now at a volume of about 1 to 1.2 liters, were added 5 cc. of N/1 acetic acid and 50 gm. of sodium acetate. A slight precipitate separated which was removed by centrifugation and discarded. 1.2 volumes of alcohol were now added to the supernatant and the precipitate was recovered by centrifugation. The precipitate was dissolved in about 250 cc. of water and 25 gm. of solid trichloracetic acid were dissolved directly in the turbid solution. After standing 2 hours at 0°, a heavy deposit of coagulated protein precipitated from solution and was separated by centrifugation. The clear, pale yellow and strongly acid supernatant liquid, containing most of the polysaccharide, was placed in the ice box. The precipitate of coagulated protein, still containing a considerable amount of adsorbed specific polysaccharide, was suspended in 100 cc. of water, cooled to 0° and brought as completely as possible into solution by the *cautious* addition of ice-cold 2 N NaOH. The solution was not allowed to become definitely alkaline at any time. A solu-

tion of 50 per cent trichloracetic acid was then carefully added until the point of maximum precipitation was reached. After standing 10 minutes, the coagulated protein was centrifuged and the supernatant liquid was added to that in the ice box. Solution and reprecipitation of the coagulated protein were repeated until the supernatant fluid gave only a faint or a negative Molisch test. A total of two or three reprecipitations sufficed. To the combined supernatant liquids containing the specific polysaccharide in a volume of about 500 cc., 20 gm. of sodium acetate were added and the solution was cooled to 0°. On the addition of 1.2 volumes of alcohol to the chilled solution, the polysaccharide precipitated as a white flocculent mass. The substance was collected in the usual way and dissolved in 100 cc. of water. After adjusting the reaction to approximately pH 4 and allowing the solution to stand for several hours, a small amount of insoluble residue was separated by centrifugation. 5 gm. of sodium acetate were added and the polysaccharide was precipitated by adding an equal volume of cold alcohol. The precipitate was separated, and dissolved in 75 cc. of water, yielding a clear and almost colorless solution which gave none of the usual protein tests. The material was made definitely acid to Congo red with HCl, and was dialyzed until no chlorine ions were detectable in the dialysate. A small quantity of deacetylated specific polysaccharide which had precipitated from the solution was separated by centrifugation. The viscous solution of acetyl polysaccharide was poured into 10 volumes of acidulated acetone. The precipitated carbohydrate was separated by filtration and washed with alcohol and ether. 2.1 gm. were recovered. The product thus obtained was nearly white in color. A 1 per cent solution gave no test for protein.

3. Analytical Methods.—The acetyl polysaccharide was analyzed for total nitrogen by a modification of the Pregl (16) method. Amino nitrogen was determined by the method of Van Slyke (17). Acetyl determinations were made on samples of 15 mg. by Pregl's method (18). The specific optical rotation was observed in a 2 dm. tube on aqueous solutions of known concentration. The acid equivalent of the specific acetyl polysaccharide was determined by titrating an aqueous solution of known concentration at 0° to pH 7 with N/50 NaOH. The acid equivalent of the deacetylated carbohydrate was determined by dissolving a weighed sample in a known quantity of N/10 HCl, then neutralizing the measured HCl with an equivalent quantity of N/10 NaOH, and finally titrating the finely suspended isoelectric polysaccharide to pH 7 with N/50 NaOH. Reducing sugars were determined by the Hagedorn-Jensen method (19) after hydrolyzing solutions of known concentration with 1.5 N HCl in sealed tubes at 100°.

II. Chemical Properties of the Acetyl Polysaccharide of Pneumococcus Type I

By the methods described above, in which treatment with alkali was purposely avoided, the soluble specific substance of Pneumococcus Type I has been isolated in the form of an ash-free acetyl polysaccha-

ride possessing marked acidic properties. The specific carbohydrate in this form is very soluble in water, and gives solutions of high viscosity. Aqueous solutions show a specific optical rotation of about +270°. The naturally acetylated polysaccharide contains 4.85 per cent of nitrogen, approximately one-half (45 per cent) of which is liberated in the amino form when the substance is treated with nitrous acid in the cold. It does not reduce Fehling's solution until after hydrolysis with dilute mineral acids. At the same time that reducing sugars appear in the solution, the serological specificity of the acetyl polysaccharide is destroyed. The behavior in this respect is identical with that of the deacetylated polysaccharide.

On the addition of hydrochloric acid-napthoresorcinol, both forms of the specific carbohydrate give a marked color reaction indicating the presence of uronic acids. In addition to being soluble in water, the acetyl polysaccharide is soluble in 80 per cent acetic acid. Aqueous solutions of 0.5 per cent concentration are precipitated by the following reagents: phosphotungstic acid, silver nitrate, neutral and basic lead acetate; and are incompletely precipitated by barium hydroxide. Unlike the deacetylated product, the acetyl polysaccharide is precipitated by tannic acid but not by uranyl nitrate. It gives no color reaction with iodine-potassium iodide solution. Weak solutions of potassium permanganate are not immediately decolorized by the acetyl polysaccharide. The biuret, ninhydrin, sulfosalicylic and picric acid tests are negative. No traces of phosphorus or sulfur were detectable in the most highly purified preparations of the specific acetyl polysaccharide.

The following experiments were carried out, in order to identify the organic acid liberated from the naturally acetylated polysaccharide by alkaline hydrolysis, and to determine whether the chemical properties of the carbohydrate after deacetylation are identical with those of the specific polysaccharide formerly obtained by methods involving the use of alkali in the process of isolation.

1. Conversion of the Acetyl Polysaccharide to Its Deacetylated Derivative by Alkaline Hydrolysis

0.0693 gm. of acetyl polysaccharide (Preparation 2, Table I) was dissolved in 7 cc. of water and neutralized to phenolphthalein with 1.42 cc. of N/10 NaOH. 7 cc. more of N/10 alkali were added and the mixture was heated in a boiling water

bath for 35 minutes. The reaction mixture was then neutralized and finally 1.5 cc. of N/10 HCl were added in excess. A precipitate appeared in the solution. The flask containing the mixture was placed in the ice chamber for 24 hours, after which time the precipitate was separated by centrifugation and washed several times with small quantities of ice water. 0.052 gm. of material was recovered. The substance was dried at 100° in high vacuum.

TABLE I

Analyses of the Acetyl Polysaccharide of Pneumococcus Type I

Preparation No.	Source	Acid equivalent	Specific rotation	Ash	C	H	Total nitrogen	Amino nitrogen	Acetyl	Phosphorus	Reducing sugars after hydrolysis	Highest dilution of polysaccharide reacting with antipneumococcus serum
				per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
1	Bacterial cells	—	+270°	0.0	—	—	4.89	2.30	5.9	0.0	—	1:5,000,000*
2	" "	—	+265°	0.0	—	—	4.86	2.21	6.9	—	—	1:5,000,000*
3	Autolyzed broth cultures	576	+277°	0.0	42.55	6.58	4.85	2.22	6.0	0.0	32.0	1:5,000,000*
2 A, deacetylated	†	535	+297°	0.0	‡	‡	5.05	2.50	0.0	—	27.6	1:5,000,000§

* Type I antipneumococcus serum previously absorbed with Preparation 2 A (deacetylated).

† This sample of deacetylated polysaccharide was obtained by alkaline hydrolysis of Preparation 2. This material is identical with the carbohydrate formerly known as the soluble specific substance of Type I Pneumococcus.

‡ An analysis of carbon and hydrogen was made on a sample of deacetylated carbohydrate which had been reprecipitated five times at its isoelectric point. The material contained no ash, and had a carbon content of 40.33 per cent and a hydrogen content of 6.23 per cent.

§ Unabsorbed Type I antipneumococcus serum.

The analytical data presented in Table I show that the deacetylated product (Preparation 2 A) obtained by alkaline hydrolysis of the acetyl polysaccharide (Preparation 2) contains no acetyl groups[1] and is in all respects chemically identical with the polysaccharide which has hitherto been known as the soluble specific substance.

[1] Heidelberger and Kendall have previously found that the Type I polysaccharide (deacetylated) contains no acetyl groups. (See *J. Exp. Med.*, 1931, **53**, Table III on page 636.)

2. Identification of Acetic Acid in the Acetyl Polysaccharide of Pneumococcus Type I

0.80 gm. of Preparation 3 (Table I) was dissolved in 25 cc. of water and neutralized. 25 cc. of N/1 NaOH were added, and the solution was heated in a boiling water bath for 40 minutes. The mixture was cooled and 6 cc. of 5 N H_2SO_4 were added. The solution was placed in a Claissen flask and distilled *in vacuo.* The delivery tube of the Claissen flask was so bent that it extended to the bottom of a receiving flask. The latter contained a suspension of freshly prepared and carefully washed silver carbonate, and the entire flask was packed in ice. The contents of the Claissen flask was distilled nearly to dryness, then 50 cc. of water were added and the distillation repeated. This was done three times in all. The receiving flask was then disconnected, and the excess silver carbonate was removed by filtration. The filtrate from the silver carbonate, which contained the silver salt of a volatile organic acid, was concentrated to 20 cc. *in vacuo.* The solution was warmed slightly and filtered. The filtrate was cooled to 0°, and 50 cc. of neutral, freshly distilled ethyl alcohol were slowly added. A snow-white product crystallized promptly from the solution. In crystalline form this material was identical with an authentic sample of silver acetate. The unknown silver salt was filtered, washed with a little dilute alcohol and dried *in vacuo* at 56° to constant weight; 0.080 gm. was recovered. The product was analyzed for silver by the usual method. The silver was weighed as silver iodide.

Analysis: 0.0483 gm. substance gave 0.0677 gm. AgI.

CH_3COOAg Calculated: Ag 64.7 per cent.
Found: Ag 64.4 per cent.

From the above analysis it is seen that the volatile organic acid liberated from the polysaccharide by alkaline hydrolysis is acetic acid, bringing chemical proof that the specific carbohydrate exists as an acetyl polysaccharide. The exact mode of linkage of the acetic acid is not as yet definitely known. Judging from the ease with which the acetyl groups are removed by dilute alkali, even at room temperature, it seems certain that the nitrogenous groups of the polysaccharide are not acetylated. It appears more likely that in the native polysaccharide the acetic acid is bound directly to the hydroxyl groups attached to a carbon atom.

The possibility remains, of course, that in addition to the acetyl groups, the specific carbohydrate, in the state in which it exists as a natural constituent of the capsular substance, may also have other labile groups attached to it. However, there is certain evidence which lends support to the view that the only chemical difference between

the naturally acetylated and the artificially deacetylated polysaccharide lies in the presence of acetyl groups in the former substance. For it has been found that the acid equivalent of the acetyl polysaccharide is 576, while that of the deacetylated substance is 535, when each is titrated to pH 7 with N/50 NaOH. The difference in the observed values of the acid equivalent is 41, which is in close agreement with the theoretical value of 43, representing the difference required if the native polysaccharide contains one acetyl group per 576 of formular weight. The specific polysaccharide may therefore be regarded, tentatively at least, as an acetyl ester.

The chemical evidence thus far available indicates that the soluble specific substance of Pneumococcus Type I exists in its native state as an acetyl polysaccharide, and that the specific substance recovered by the earlier methods must now be regarded as the deacetylated derivative of the native carbohydrate from which the labile acetyl groups have been removed by treatment with alkali during the chemical manipulations incident to its isolation.

In the following experiments the immunological properties of the two forms of the specific polysaccharide and their serological relationships to each other will be considered in terms of these chemical findings.[2]

III. Immunological Properties of the Acetyl and the Deacetylated Polysaccharide of Pneumococcus Type I

The preceding experiments have revealed the chemical relationship existing between the naturally acetylated and artificially deacetylated form of the specific capsular polysaccharide of Pneumococcus Type I. In the following experiments these chemical differences are shown to be reflected in the immunological reactions of the two forms of the specific carbohydrate. The acetyl polysaccharide is found not only to fulfil all the immunological criteria of type specificity, but also to possess certain additional specific properties which the deacetylated polysaccharide lacks through the loss of the extremely labile acetyl groups.

[2] The authors wish to express their appreciation to Mr. Frank H. Babers for his kindly cooperation in much of the analytical work and to Dr. Michael Heidelberger and Dr. P. A. Levene for their interest and helpful suggestions.

1. Precipitation of the Acetyl and Deacetylated Polysaccharide in Absorbed and Unabsorbed Type I Antipneumococcus Serum

Immune horse serum[3] was absorbed by the fractional addition of a 1:2000 solution of the acetyl polysaccharide, until the supernatant serum after removal of the successive precipitates no longer reacted on the further addition of the specific substance. An equal portion of the same lot of serum was similarly absorbed with the deacetylated polysaccharide.

10 cc. of antipneumococcus horse serum (Type I) were diluted with 8 cc. of salt solution. To the diluted serum, 1 cc. of 1:2000 solution of the specific polysaccharide was added. The mixture was incubated for 2 hours at 37° and then placed in the ice box for the same length of time. The precipitate was thrown down by centrifugation in the cold and the clear supernatant serum was pipetted off. This procedure was repeated three times in all. Finally, 0.5 cc. of the solution of polysaccharide was added to the serum and the mixture was incubated for 2 hours at 37° and then allowed to stand in the ice box 24 to 48 hours. After removal of the final traces of precipitate by centrifugation in the cold, the clear supernatant serum was pipetted off and made up to a volume of 25 cc. with salt solution, so that each 0.5 cc. of absorbed serum used in the tests equalled 0.2 cc. of original serum.

The original serum and the two separately absorbed portions of the same serum were tested for the presence or absence of precipitins for both forms of the capsular polysaccharide. The results of the precipitin tests are given in Table II.

From the data recorded in Table II it is seen that both the acetyl and the deacetylated polysaccharide were precipitated by the original, unabsorbed serum in the highest dilution tested, representing a final concentration of 1 part in 3 million. The serum absorbed with the deacetylated polysaccharide, after removal of all precipitins for this form of the specific carbohydrate, still reacted with the acetyl polysaccharide in equally high dilution. On the other hand, after absorption with the acetyl polysaccharide, the serum was completely exhausted of all precipitins for both forms of the carbohydrate, as shown by the lack of reaction when tested with each substance in dilutions ranging from 1:20,000 to 1:3,000,000. It is a significant fact that the

[3] The antipneumococcus horse serum used in these experiments was provided through the courtesy of Dr. Augustus Wadsworth, Director of the Division of Laboratories and Research, New York State Department of Health, Albany.

deacetylated polysaccharide selectively takes out from the serum only the precipitins for itself, whereas the acetyl polysaccharide completely removes all the precipitating antibodies for both forms of the specific substance.

Enders (8) (1930) demonstrated that there exists in the autolytic products of Pneumococcus Type I a substance which is specifically precipitable in immune serum devoid of all antibodies for the soluble specific substance (deacetylated). He further showed that this material after being heated in a weakly alkaline solution lost its capacity to react in the same serum. The substance therefore appeared to be so sharply differentiated by its immunological reactions and its instability to alkali, that Enders considered it to be quite distinct, and provisionally called it "the A substance" to distinguish it from the specific carbohydrate. Wadsworth and Brown (11, 12) (1931, 1933) isolated from the bacterial cells a substance which, like the A substance of Enders, precipitated with Type I antipneumococcus serum that had previously been absorbed with the soluble specific substance (deacetylated). They also found that the substance designated by them "the cellular carbohydrate," when boiled for 2 minutes in N/100 NaOH no longer reacted in the absorbed serum, indicating, as the authors suggest, that boiling in alkaline solution had so altered their original material that its activity under these conditions approximated that of the soluble specific substance (deacetylated).

TABLE II

Precipitation of the Acetyl and Deacetylated Polysaccharide of Pneumococcus Type I in Homologous Antiserum before and after Absorption of the Anti-carbohydrate Precipitins

Antipneumococcus Serum Type I	Acetyl polysaccharide					Deacetylated polysaccharide				
	1:20,000	1:100,000	1:500,000	1:1,000,000	1:3,000,000	1:20,000	1:100,000	1:500,000	1:1,000,000	1:3,000,000
Unabsorbed	++++	++++	+++±	+++	++	++++	++++	++++	+++	++
Absorbed with deacetylated polysaccharide	++++	++++	++±	++	+	–	–	–	–	–
Absorbed with acetyl polysaccharide	–	–	–	–	–	–	–	–	–	–

++++ = complete precipitation, compact sediment with clear supernatant.
– = no precipitate formed, fluid clear.

The final readings were made after incubating the reacting mixtures 2 hours at 37° and overnight in the ice box.

The specific precipitation of the acetyl polysaccharide in serum previously absorbed with the deacetylated carbohydrate, and the readiness with which the former substance is converted into the latter by alkali and heat, are similar to the relationships observed by Enders (8), and by Wadsworth and Brown (12), between the substances isolated by them and the soluble specific substance which they prepared according to methods previously described in this laboratory. Since the specific substance thus prepared is now known to be the deacetylated polysaccharide, it seems not improbable that the differences they observed, like those noted in Table II, represent the reactions not of two different carbohydrates but of a single substance in two chemically different forms; namely, the naturally acetylated and the artificially deacetylated polysaccharide.

2. *Agglutination of Type I Pneumococci in Homologous Antiserum before and after Absorption with the Acetyl and Deacetylated Polysaccharide*

Type I antipneumococcus serum was separately absorbed with the acetyl and the deacetylated polysaccharide as previously described. The results of the agglutination tests of Type I pneumococci in homologous antiserum before and after specific absorption are given in Table III.

The experimental data presented in Table III show that absorption of Type I antiserum with the acetyl polysaccharide completely removed all the type-specific agglutinins, as evidenced by the fact that, after absorption, the serum no longer agglutinated the homologous organisms. On the other hand, the serum similarly absorbed with the deacetylated polysaccharide still agglutinated the bacteria although the titer of agglutinins was considerably reduced. The fact that after absorption with the deacetylated polysaccharide the precipitin titer appeared undiminished for the acetyl polysaccharide, while the titer of agglutinins for the bacterial cells was reduced, may be attributed not to essential differences in the antibodies involved in the two forms of immune reactions, but to differences in the technical procedures of diluting the antiserum in the agglutination test, and of maintaining an excess of serum throughout the range of the precipitin titration. Of special significance in the present study is the fact

that the acetyl polysaccharide by itself completely exhausted the serum of all demonstrable type-specific precipitins and agglutinins.

Sabin (10) and Enders (9) previously demonstrated that Type I antiserum after absorption with the specific carbohydrate (deacetylated) still agglutinated pneumococci of the homologous type. Enders further showed that when the bacterial cells were heated in a weakly alkaline medium, they lost the capacity to react in the absorbed serum although in this altered state they were still specifically agglutinated by the original, unabsorbed serum. The fact that the bacteria were agglutinated by immune serum containing no antibodies reactive with the specific carbohydrate (deacetylated), and the further observation that after boiling for

TABLE III

Agglutination of Type I Pneumococci in Homologous Antiserum before and after Absorption with the Acetyl and the Deacetylated Polysaccharide

Antipneumococcus Serum Type I	Agglutination of Pneumococcus Type I in serum dilutions						
	1:10	1:20	1:30	1:40	1:60	1:80	1:100
Unabsorbed	++++	++++	++++	++++	+++	++	+
Absorbed with deacetylated polysaccharide	++++	++	+±	±	–	–	–
Absorbed with acetyl polysaccharide	–	–	–	–	–	–	–

++++ = complete agglutination, compact sediment with clear supernatant.
– = no agglutination.

10 minutes at pH 8.8 the cells no longer reacted in this same serum, led Enders to the conclusion that there exists a type-specific substance distinct from the specific carbohydrate in Pneumococcus Type I.

As pointed out earlier in this paper, the acetyl polysaccharide is readily converted into the deacetylated substance by treatment with alkali. This fact, together with the observations just cited on the serological reactions of the two forms of the specific polysaccharide, not only substantiates the findings of the former investigators but also furnishes evidence of the immunological significance of this hitherto unrecognized relationship. Thus, on the basis of the present evidence, it appears that the acetyl polysaccharide represents the soluble specific substance in a form that fulfils all the serological requirements

of type specificity without the necessity of predicating a second substance distinct from the specific carbohydrate itself. These observations are further confirmed by the results of the following protection experiments.

3. Protective Action of Type I Antipneumococcus Serum before and after Absorption with the Acetyl and Deacetylated Polysaccharide

Protection tests in mice were carried out according to the method described by Felton (15).

Dilutions of the unabsorbed and absorbed serum, calculated on the basis of original serum volume, were made, ranging from 1:10 to 1:500. 0.5 cc. of each dilution of serum together with 0.5 cc. of 1:200 dilution of a 12 hour plain broth culture of Pneumococcus Type I was injected intraperitoneally into white mice weighing from 18 to 21 gm. The virulence of the organisms was such that 10^{-8} cc. of culture caused the death of normal control mice in 48 hours.

All mice alive and well 7 days after inoculation were considered effectively protected and were recorded as survivals.

The results of experiments to determine the protective action of Type I antipneumococcus serum before and after absorption with the acetyl and deacetylated polysaccharide are given in Table IV.

The outcome of the protection tests (Table IV) shows that after absorption with the deacetylated polysaccharide, the serum still possessed protective action, although the titer of protective antibodies was considerably reduced. In an earlier study of the neutralizing effect of the soluble specific substance, Sabin (10) showed that this substance, which in the light of the present results was presumably in the form of the deacetylated polysaccharide, only partially neutralized the protective power of Type I antipneumococcus serum He attributed the residual protective action of the absorbed serum to the presence of type-specific antibodies not neutralized by the homologous soluble specific substance and distinct from the anticarbohydrate precipitins. The comparative data presented in Table IV show that the soluble specific substance in the form in which it naturally occurs as the acetyl polysaccharide completely removed the protective antibodies in Type I antiserum. This neutralizing effect is shown by the fact that after removal of all the anticarbohydrate precipitins by absorption with the acetyl polysaccharide, the immune serum was de-

void of protective action when titrated by the method employed in the present experiments.

These results again emphasize the relationship existing between the natural acetyl polysaccharide and its deacetylated derivative. It now becomes apparent why the specific carbohydrate in the form in which

TABLE IV

Protective Action in Mice of Type I Antipneumococcus Serum before and after Absorption with the Acetyl and the Deacetylated Polysaccharide of Pneumococcus Type I

Dilution of serum	Antipneumococcus Serum Type I					
	Unabsorbed		Absorbed with			
			Deacetylated polysaccharide		Acetylpolysaccharide	
1:500	S	S	D 21	D 28	D 22	D 25
1:250	S	S	D 26	D 51	D 20	D 23
1:100	S	S	S	S	D 17	D 18
1:50	S	S	S	S	D 17	D 21
1:10	S	S	S	S	D 19	D 23

All mice were injected intraperitoneally with 0.5 cc. of diluted serum together with 0.5 cc. of 1:200 dilution of broth culture of Pneumococcus Type I.

Controls

Pneumococcus Type I	Mice receiving no serum
cc.	
10^{-6}	D 45
10^{-7}	D 45
10^{-8}	D 48

S = survived. D = died. Numerals indicate the number of hours elapsing before death of the animal.

it was first isolated was later found to be deficient in certain specific properties, notably in its failure to absorb completely the type-specific antibodies from immune serum. By the methods employed in the original isolation, the specific carbohydrate is now known to have been artificially deacetylated, and its immunological deficiencies have been found to be associated with the loss of the highly reactive but ex-

tremely labile acetyl groups. The significance of this fact is made evident by the results of the preceding experiments, in which it has been shown that the acetyl polysaccharide with these chemical groups intact specifically bound and completely removed from the serum all the type-specific antibodies.

4. Antigenic Action of the Acetyl Polysaccharide in Mice

In order to determine whether the acetyl polysaccharide is capable of inducing active immunity against infection with pneumococci of the homologous type, the antigenicity of this form of the specific carbohydrate was tested in mice and its action compared with that of the deacetylated polysaccharide

Six mice were given three intraperitoneal injections, at 3 day intervals, of 0.5 cc. of 1:2 million dilution of Type I acetyl polysaccharide; another group of six mice was similarly treated with identical amounts of the deacetylated carbohydrate prepared by heating the original material in N/20 alkali for 30 minutes at 100°. 6 days after the last immunizing injection both groups of mice were infected by the intraperitoneal injection of a virulent culture of Pneumococcus Type I in amounts ranging from 10^{-5} to 10^{-7} cc., the maximum number of infecting organisms being 1000 times greater than that which proved fatal in the normal control mice.

The results of the experiments on the active immunization of mice with both forms of the specific carbohydrate are given in Table V.

As shown in Table V, the mice which had received in divided doses an amount of acetyl polysaccharide totaling only 0.00075 mg. of specific substance survived the injection of an amount of virulent culture of Pneumococcus Type I greatly in excess of that causing fatal infection in the untreated control animals. Repetitions of this test in mice have shown that the active immunity induced by the acetyl polysaccharide is strictly type-specific, affording no protection against infection with pneumococci of the heterologous Types II and III. It is equally clear from the results of this and other similar experiments that the deacetylated polysaccharide is wholly devoid of antigenic action. This total lack of immunizing effect is all the more striking in this particular instance, since the deacetylated substance was derived from the originally active acetyl polysaccharide by

merely heating the latter in alkaline solution—a procedure previously shown to deprive the native carbohydrate of its acetyl groups.

During the past ten years a number of investigators using various methods have recovered from Pneumococcus, substances which have been shown to possess the property of inducing active immunity in mice against infection with organisms of the homologous type. In many instances, the antigenic and serological behavior of these substances was so distinct that the authors designated them by special terms in order to distinguish them from the soluble specific substance (deacetylated). Thus, there are now current in the literature

TABLE V

Active Immunity Induced in Mice by the Acetyl and Deacetylated Polysaccharide of Pneumococcus Type I

Amount of culture Pneumococcus Type I	Normal mice controls (untreated)	Mice receiving 3 injections of 0.5 cc. of 1·2 million solution of			
		Acetyl polysaccharide Type I		Deacetylated polysaccharide Type I	
cc.					
10^{-5}	—	S	S	D 68	D 68
10^{-6}	D 44	S	S	D 34	D 44
10^{-7}	D 52	S	S	D 58	D 76
10^{-8}	D 93	—	—	—	—

The treated mice were infected 6 days after the last immunizing injection.

— = not done.

S = survived. D = died. Numerals indicate the number of hours elapsing before death of the animal.

descriptive terms such as the following: "the water-soluble fraction" of Perlzweig and his coworkers; "the A substance" of Enders; "the cellular carbohydrate fraction" of Wadsworth and Brown, and "the non-polysaccharide and probably non-protein derivative" of Felton. With the possible exception of the A substance, which Enders did not test for antigenicity in mice, these various cell derivatives have been found to produce type-specific immunity in this particular species of animal.

Since it is obviously impossible within the scope of this paper to review the individual contributions in detail, brief reference will be made only to those studies concerned with the antigenicity of specific fractions derived from Pneumococcus Type I.

Perlzweig and Steffen (4) (1923) extracted from the bacterial cells a water-soluble fraction which induced specific immunity in mice. This observation was later confirmed by Ferry and Fisher (20) (1924, 1925) who obtained from washings of the organisms an aqueous extract which had similar antigenic properties in mice. The water-soluble antigen of Perlzweig and Steffen proved resistant to the prolonged action of autolysis and tryptic digestion. They further pointed out the suggestive fact that boiling the antigenic material for 5 minutes in alkaline solution (pH 9) destroyed its immunizing action in mice, while similar exposure to heat in a slightly acid medium (pH 6) did not impair its antigenicity. Perlzweig and Keefer (5) (1925) recovered from the filtrate of broth cultures a substance which, like that derived from the cells, produced active immunity in mice. Although Perlzweig and his coworkers regarded the immunizing material as protein in character they pointed out evidence suggestive of its non-protein nature.

Schiemann and his collaborators (6, 7) (1927, 1931) first brought convincing evidence that the type-specific polysaccharide of Pneumococcus Type I, in the form isolated by them, produced specific immunity when injected in relatively minute amounts into mice. They also found that if administered to mice in larger doses, this form of the specific carbohydrate not only failed to incite immunity but on the contrary was often toxic and caused purpura. Wadsworth and Brown (11, 12) (1931, 1933) isolated from the bacterial cells a specific fraction designated by them "the cellular carbohydrate." This substance corresponded in its antigenic and purpura-producing action to the carbohydrate of Schiemann and Casper (6), and was similar in its immunological reactions to the A substance of Enders (8). Felton (14) (1932) isolated from Pneumococcus Type I a substance inducing type-specific immunity in mice which from its chemical properties he concluded was "a non-polysaccharide and probably non-protein derivative" of the bacterial cells.

The consistently negative results of all former attempts in this laboratory to induce active immunity in mice with the specific carbohydrate are now known to have been due to the fact that the polysaccharide was then used only in its deacetylated form. As shown in Table V, the change from the antigenic to the non-antigenic form of the carbohydrate is brought about whenever the originally active acetyl polysaccharide is converted by alkali into its deacetylated derivative. This difference in antigenic action, like that already noted in the serological behavior of the two forms of the polysaccharide, is referable to known differences in chemical constitution.

An analysis of the specific reactions of the acetyl polysaccharide discloses a previously unsuspected similarity between this form of the specific carbohydrate and the antigenically active fractions described

by other investigators. From the chemical and immunological properties of the acetyl polysaccharide it seems highly probable that this substance in the purified state accounts for the antigenic action of the carbohydrate of Schiemann and Casper (6) and of Wadsworth and Brown (11, 12). As in the case of these substances, the acetyl polysaccharide is antigenically effective in mice only when administered in extremely minute quantities. Although an extensive study of the purpura-producing action of the acetyl polysaccharide has not been made, in several instances purpura has been noted in mice injected with amounts of this substance ranging from 0.4 to 4.0 mg.

That the antigenic action of the water-soluble fraction of Perlzweig and his coworkers (4, 5) may have been due to the presence of traces of unhydrolyzed acetyl polysaccharide seems not unlikely from the readiness with which it lost its immunizing capacity when heated in alkaline solution.

As pointed out earlier, the A substance of Enders (8) and the cellular carbohydrate of Wadsworth and Brown (11, 12) correspond in their serological reactions to those of the acetyl polysaccharide. In addition, both of these substances were shown to be equally sensitive to the destructive action of alkali. While it cannot be stated with certainty that these substances are identical, their properties parallel those of the acetyl polysaccharide so closely that it seems probable that their biological activity is due to this substance.

5. *Antigenic Action of the Acetyl Polysaccharide in Rabbits*

Despite the number of observations on the antigenicity in mice of specific fractions derived from Pneumococcus Type I, comparatively little work has been done to determine the capacity of these substances to incite antibody formation in rabbits. The following experiments, therefore, were carried out to ascertain whether the acetyl polysaccharide possesses the property of stimulating the production of type-specific antibodies in rabbits.

Six rabbits were given intravenous injections of acetyl polysaccharide daily for 6 days, followed by a rest period of 1 week. Three courses of injections were given in all. Two rabbits received 1 cc. of 1:1000 solution of the substance; two others were injected with 1 cc. of 1:10,000 solution; the remaining two were given 1 cc. of 1:100,000 solution. At the end of the third course of injections, each of

the three groups of rabbits had received a total quantity of acetyl polysaccharide amounting to 18 mg., 1.8 mg. and 0.18 mg. respectively. 7 days after the second and third series of injections, test bleedings were made and the sera were tested for the presence of type-specific agglutinins, precipitins and protective antibodies.

The detailed protocols of the serological tests are omitted, since in no instance were type-specific agglutinins, precipitins or protective antibodies demonstrable in the serum of the rabbits which had previously received repeated injections of Type I acetyl polysaccharide.

Ten days after the last course of injections, each rabbit was infected by the intradermal injection of 0.2 cc. of undiluted blood broth culture of Pneumococcus Type I according to the method described by Goodner (21). The infected rabbits developed at the site of inoculation typical lesions characterized by areas of massive edema and hemorrhagic necrosis. All six of the animals died within 48 to 96 hours after the onset of infection. In no instance, therefore, was there any evidence of increased resistance brought about by the prolonged series of injections of Type I acetyl polysaccharide in amounts totaling 0.18 to 18 mg. In view of the fact that only minute amounts of the acetyl polysaccharide were effective in evoking an immune response in mice, it is conceivable that these rabbits were given too large doses; however, in terms of body weight, the dosage in those rabbits that had received a total of 0.18 mg. was presumably within the range of the amounts found to be effective in mice.

In view of these results, it is significant that the presence of the acetyl polysaccharide was demonstrated by the precipitin reaction in the serum of these rabbits 7 days after the second and third course of injections. This observation indicates that the acetyl polysaccharide is only very slowly excreted, and indirectly suggests that no antibodies were formed, otherwise the substance would in all probability have disappeared more rapidly from the circulation. That the acetyl polysaccharide is actually excreted as such by the kidney and appears in the urine in this specifically reactive form was shown in the case of two other rabbits. One of these animals was given a single large dose of 17 mg. of active substance intravenously and the other a similar amount intraperitoneally. Samples of urine from these animals were collected and tested for the presence of the acetyl polysaccharide. Specific precipitation occurred in the urine of both rabbits

on the addition of Type I antipneumococcus serum from which all antibodies reactive with the deacetylated polysaccharide had been previously removed by specific absorption. The specificity of this test conclusively demonstrates that the polysaccharide was excreted in the acetylated form. The urine of both rabbits still showed the presence of the acetyl polysaccharide, as demonstrated by the specific precipitin reaction, 7 days after injection, at which time the observations were discontinued.

Under the conditions of this experiment, the acetyl polysaccharide failed to induce any immune response in rabbits. The serum of the treated animals contained no demonstrable antibodies, and the animals themselves were not protected against subsequent infection with organisms of the homologous type. Moreover, it has been shown that the acetyl polysaccharide persisted in the circulation of the treated rabbits for considerable periods of time, was slowly excreted by the kidney and appeared in the urine in its naturally acetylated form.

It is of course possible that in the present instance the failure of the acetyl polysaccharide to induce antibody formation or to incite active immunity may be attributed to the inadequate number of rabbits tested or to the use of improper amounts of the substance. No assumption is made as to the difference in the antigenic action of the acetyl polysaccharide in mice and in rabbits. The explanation must await further study of this interesting and significant phase of the problem.

DISCUSSION

So far as is known, the only chemical difference between the acetyl polysaccharide and its deacetylated derivative lies in the presence or absence of the acetyl groups. Evidence in support of this view is the difference observed in the acid equivalents of the two forms of the specific carbohydrate. On alkaline hydrolysis there is liberated from the acetyl polysaccharide approximately 6 per cent of acetic acid which is organically bound in the intact molecule in the form of an acetyl ester. From solutions of the acetyl polysaccharide that have been treated with alkali, the deacetylated carbohydrate has been recovered and the substance thus derived has been found to correspond in chemical and serological properties to the polysaccharide formerly known as the "soluble specific substance."

The acetyl polysaccharide possesses all the specific immunological characteristics of the deacetylated derivative and in addition exhibits other distinctive properties. In highly purified form the acetyl polysaccharide, in contradistinction to the deacetylated substance, completely absorbs all demonstrable type-specific antibodies from antiserum of the homologous type; it induces active immunity and incites purpura in mice; it is specifically precipitable in immune serum from which the type-specific anticarbohydrate precipitins reactive with the deacetylated polysaccharide have been removed by specific absorption; it is extremely unstable to the action of alkali.

The results of the present study offer an explanation of many of the perplexing problems that have arisen concerning the nature and specific properties of the soluble specific substance. One of these is the question of the antigenicity of the specific carbohydrate. In the form in which it was originally isolated the polysaccharide was found to be devoid of antigenic action in mice and in rabbits, and considerable evidence was presented that this substance functioned only as a hapten. However, a number of investigators (4, 5, 14, 20) have isolated substances, in some instances of undoubted carbohydrate nature (6, 12), which were antigenic, inducing type-specific immunity in mice. The present experiments show that minute quantities of the purified acetyl polysaccharide give rise to active immunity in mice. While it is impossible to state that the antigenic activity of the specific fractions isolated by others is due to the presence of the acetyl polysaccharide in the preparations employed, this possibility seems not unlikely. The differences between the antigenic and nonantigenic forms of the specific carbohydrate are thus related to known differences in chemical constitution. The antigenicity of the acetyl polysaccharide, in mice at least, is intimately associated with the presence of the acetyl groups in the polysaccharide molecule.

The writers have never maintained that complex carbohydrates may not function as antigens, but until the present experiments with the highly purified acetyl polysaccharide they had obtained no evidence in experimental animals that this was the case. Many years ago, Ulenhuth (22) (105) presented evidence of the antigenic action of gum arabic, pointing out that this was the first time that specific antibodies had been demonstrated in the serum of animals immunized

with a carbohydrate. Recently in collaboration with Remy, Uhlenhuth (23) (1933) has confirmed his early observations, showing that after prolonged immunization with gum arabic, the serum of the treated rabbits contained specific precipitins and complement-fixing antibodies. Ford (24, 25) (1906–07) found that the serum of rabbits immunized with extracts of *Amanita phalloides* and *Rhus toxicodendron*, possessed marked antihemolytic and antitoxic properties. The active principle of these extracts was isolated and identified in each instance as a glucoside.

A question that has been difficult of interpretation is that relating to the purpura-producing activity of the specific carbohydrate. Specific substances of carbohydrate nature isolated by other workers have been found to incite purpura in mice, while the polysaccharide in the form originally isolated does not possess this activity. However, the fact that the mere presence of acetyl groups in a physiologically active substance may greatly modify its activity is well known in the case of acetyl choline which has at least one thousand times the activity of the parent base (26). It is not certain that the purpura-producing activity of the acetyl polysaccharide is solely related to the presence of these groups in the molecule. However, it is known that with loss of acetyl groups, the polysaccharide also loses the capacity to induce purpura.

Another of the perplexing problems that have arisen has been the possibility, indicated by the work of several investigators, that the specific carbohydrate is not the only substance concerned in the type specificity of Pneumococcus Type I. The concept of two type-specific substances has its origin in the observation that the polysaccharide in the form first isolated does not absorb all the type-specific antibodies from immune serum (10). This fact suggested the presence in the serum of antibodies distinct from the anticarbohydrate precipitins, and the coexistence in the cell of another substance unrelated to the polysaccharide. Support for this view was found in the demonstration and subsequent isolation by others (8, 12) of a substance that was specifically precipitable in antiserum from which all precipitins for the specific carbohydrate had been removed. Several explanations were proposed, chiefly that there exist in the cell two specific sub-

stances giving rise to two distinct antibodies, both type-specific, but each reactive only with the corresponding antigen. On the other hand, in support of the view that only a single substance is responsible for type specificity Wadsworth and Brown (11) suggested that the specific carbohydrate as first isolated may be only a radical of a more complex substance. Ward (13) suggested "the possibility that the reacting substance in the autolysate is more complex and less stable than the carbohydrate--perhaps a substance intermediate between the antigenic carbohydrate compound in the intact pneumococcus and the carbohydrate itself."

From the work of Landsteiner and others it is known that the mere presence of relatively small chemical groups in an immunologically active substance exerts a determining influence on its specificity. The present study brings evidence that in Pneumococcus Type I the specific carbohydrate with the acetyl groups intact fulfils all the serological requirements of type specificity. On the other hand, when the acetyl groups are removed the resultant product retains the polysaccharide structure and the dominant type specificity of the original carbohydrate, but loses many of the specific characteristics that distinguish the naturally acetylated polysaccharide.

SUMMARY

The soluble specific substance of Pneumococcus Type I has been chemically isolated from the bacterial cells and from autolyzed cultures as an acetyl polysaccharide.

So far as could be determined by the methods employed, the acetyl polysaccharide in highly purified form absorbs from Type I antipneumococcus serum all demonstrable type-specific precipitins, agglutinins and protective antibodies.

Mice injected intraperitoneally with minute quantities of the acetyl polysaccharide develop active immunity to subsequent infection with Pneumococcus Type I. The immunity thus induced is type-specific. In several instances purpura has been observed in mice following the injection of larger amounts of the acetyl polysaccharide.

Under the experimental conditions of this study, no type-specific precipitins, agglutinins or protective antibodies were demonstrable

in the serum of rabbits following repeated intravenous injections of the Type I acetyl polysaccharide. The treated rabbits were not immune to subsequent infection with Pneumococcus Type I.

The acetyl polysaccharide is readily converted into its deacetylated derivative by treatment with dilute alkali.

The chemical and immunological properties of the deacetylated polysaccharide are identical with those of the soluble specific substance in the chemical form in which it was originally isolated; the deacetylated form of the specific carbohydrate is non-antigenic, does not produce purpura in mice, and only incompletely absorbs the type-specific antibodies from Type I antipneumococcus serum.

The immunological significance of the acetyl polysaccharide and its possible relationship to the specific substances isolated from Pneumococcus Type I by other workers are discussed.

CONCLUSIONS

The soluble specific substance of Pneumococcus Type I is now regarded, tentatively at least, as an acetyl polysaccharide. In this form it accounts adequately for all the serological phenomena of type specificity of Pneumococcus Type I.

Addendum.—During the course of publication of the present work there has appeared a paper by Pappenheimer and Enders[4] on the specific carbohydrate of Type I Pneumococcus. On the basis of elementary analysis, amino nitrogen content and specific rotation, these authors conclude that the A substance of Enders and the specific polysaccharide previously isolated in this laboratory are closely related and that the latter is possibly a hydrolytic product of the former substance.

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FURTHER OBSERVATIONS ON THE CULTIVATION OF VACCINE VIRUS FOR JENNERIAN PROPHYLAXIS IN MAN

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In 1913, Steinhardt, Israeli, and Lambert (1) demonstrated that vaccine virus is capable of multiplication in the presence of bits of viable tissue embedded in plasma. Although this work has been confirmed by a number of investigators, cultures of vaccine virus made by means of the cover-slip technique have proved of no value in the preparation of an active agent for Jennerian prophylaxis. In 1927, Carrel and Rivers (2) devised a method for the cultivation of vaccine virus in which bits of viable chick embryo tissue embedded in plasma in flasks were used. In 1928, Maitland and Maitland (3) showed that they were able to grow vaccine virus in a medium consisting of minced hen kidney suspended in a mixture of hen serum and Tyrode's solution. In 1929, Rivers, Haagen, and Muckenfuss (4) demonstrated that cells remain viable for at least 5 days in a medium made according to Maitland's directions. However, if the cells are killed by repeated freezing and thawing, the medium no longer supports the multiplication of virus.

In a further search for a simple and safe method of cultivating vaccine virus for human use, we devised a highly satisfactory medium which consists of bits of minced chick embryo tissue (0.1 gm.) suspended in Tyrode's solution (4–5 cc.) in "collar flasks." In 1930, the results obtained with cultures of a neurovaccine virus were reported (5). In 1931, we described (6) our work with a dermal strain of vaccine virus and reported that the culture virus had been successfully used for the vaccination of 3 children. In 1932, Herzberg (7) reported that he had been able to vaccinate human beings with virus cultivated in the manner described by us. Since our last report in 1931 we have continued our observations, and it seems appropriate at this time to

record certain facts that have accumulated in regard to the culture virus and its use for Jennerian prophylaxis.

One of the objects of our work on the cultivation of vaccine virus has been to obtain an active agent, free from bacteria and contaminating viruses, that will protect human beings against smallpox with the least amount of inconvenience and discomfort to the individuals vaccinated. Consequently, all cultures of virus used in this type of work have been carefully tested for the presence of bacteria and other undesirable agents.

EXPERIMENTAL

As we have been able to show (5, 6) that vaccine virus will multiply in the presence of bits of minced chick embryo tissue (0.1 gm.) and Tyrode's solution (4–5 cc.), we decided to determine what would happen to the active agent when it was cultivated in such a medium over a long period of time. This was accomplished by making transfers in series from old cultures to flasks of fresh medium at intervals of 4 or 5 days and then testing the activity of the virus in the different culture passages by means of dermal and intradermal titrations in rabbits and vaccination of human beings.

Effect of in Vitro Cultivation on the Activity of Vaccine Virus in the Rabbit

Cultures of vaccine virus were initiated, March 9, 1931, and the titer in rabbits of the active agent in the 1st set of cultures was 10^{-6}. The titer of the virus in the 19th set of cultures was 10^{-6}, in the 30th 10^{-4}, in the 60th 10^{-2}, in the 80th 10^{-2}, in the 86th 10^{-1}. From the 88th set of cultures to the 99th only the undiluted virus produced a lesion when injected intradermally. From the record portrayed in Chart 1 it can be seen that the titer of the virus in the cultures maintained a high level, around 10^{-6}, for 19 generations and then gradually fell until only the undiluted cultures in the 88th generation produced a lesion in rabbits.

With the drop in the titer of the virus in the rabbit came a change in the character of the lesions induced by the active agent. The virus in the first 15 generations upon intradermal inoculation produced large edematous lesions with hemorrhagic and necrotic centers. With

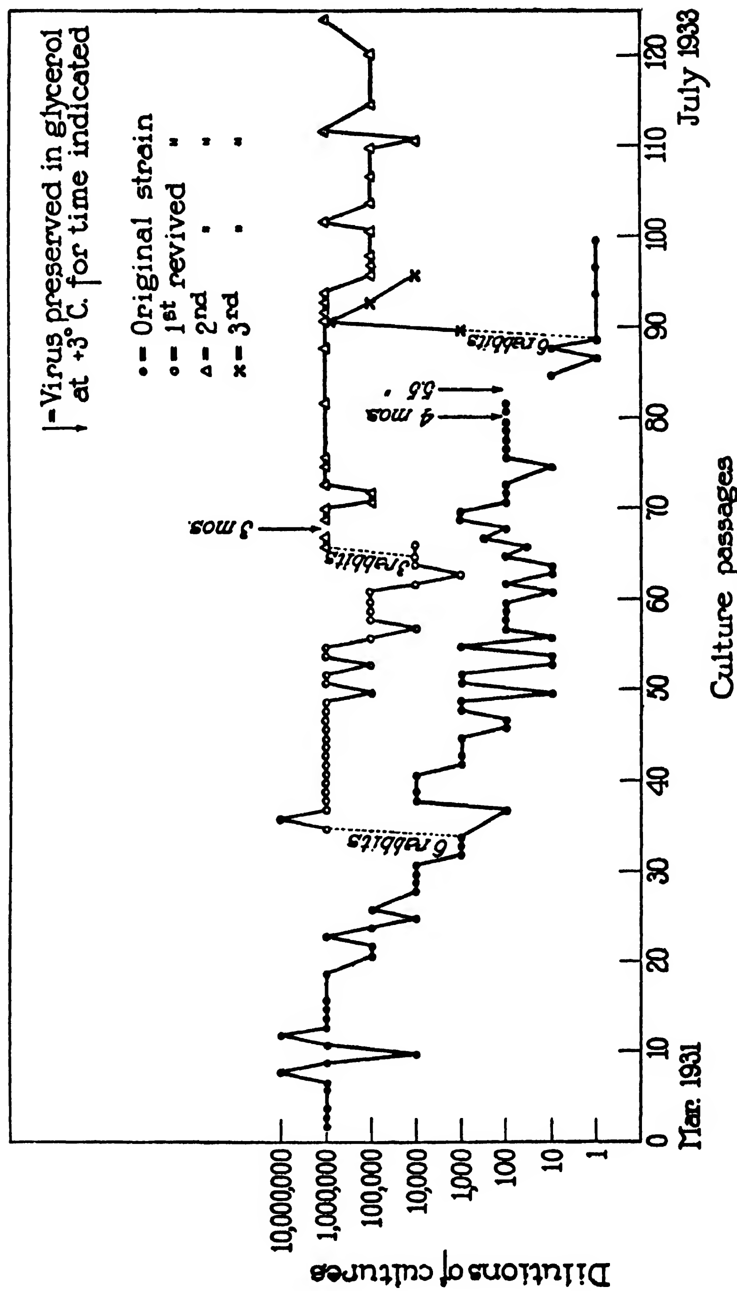

CHART 1. Graphic portrayal of the intradermal titer in rabbits of vaccine virus from different culture passages of the original 1st revived, 2nd revived, and 3rd revived strains.

material from the 15th to the 70th generations nodular lesions without hemorrhage and necrosis were excited. When the virus was spread on the scarified skin, however, typical vaccinal vesicles appeared. After the 73rd generation, only small flat red areas 1 cm. in diameter were seen at the points of intradermal inoculation of the virus. The erythema faded rapidly and was followed by a superficial scaling of the skin. Upon dermal inoculation of the virus, only a slight amount of redness and scaling was produced which was almost indistinguishable from that caused by scarification alone. In spite of the fact that extremely mild and evanescent lesions were produced in rabbits with material from these cultures, vaccine virus was present in them as will be seen when the reaction induced by them in human beings is discussed.

Revival of the Activity of Culture Virus by Means of Testicular Passages in Rabbits

Inasmuch as vaccine virus for human use is usually tested in rabbits before distribution, we have attempted to obtain a culture strain that retains its pathogenicity both for man and rabbits. Consequently, when the titer of the culture virus for the rabbit began to fall, we passed the virus through several rabbits by means of testicular inoculations. With the passaged virus new cultures were initiated. This procedure has been resorted to three times and the results are detailed below and portrayed in Chart 1.

1 cc. of the pooled 34th generation cultures was injected into each testicle of a rabbit. After 4 days the testicles were removed aseptically and an emulsion was prepared. 1 cc. of this emulsion was then injected into each testicle of another rabbit. This procedure was repeated until the virus had been passed through 6 rabbits. All of the animals had fever. The titer of the virus gradually increased and the intradermal lesions again assumed a hemorrhagic and necrotic character. With testicular virus from the 6th rabbit new cultures were initiated and have been designated as the 1st revived strain. The titer of the first 14 generations of this strain was 10^{-6}, the intradermal lesions were edematous, hemorrhagic, and necrotic. Upon further passage of the virus in cultures the titer gradually fell (Chart 1) and the character of the lesions changed from hemorrhagic and necrotic to nodular.

1 cc. of the pooled cultures of the 31st generation of the 1st revived strain was injected into each testicle of a rabbit. After 4 days the testicles were removed and

an emulsion was made. 1 cc. of this emulsion was injected into each testicle of another rabbit. This procedure was repeated until the virus had been passed through 3 rabbits. All of the animals had fever and typical vaccinal reactions in the testicles. Testicular virus from the 3rd rabbit was used to start a new set of cultures that has been designated as the 2nd revived strain. The titer of this strain has remained around 10^{-6} for 60 culture generations. Although the titer has not fallen to any great extent (Chart 1), the intradermal lesions have become less hemorrhagic and more nodular.

Repeated passages of vaccine virus in the culture medium used in this work appear to decrease the titer of the active agents for rabbits and to mitigate the severity of the lesions produced. We have been able in the manner described above to revive the culture strains by testicular passages in rabbits, and the 2nd revived strain seems to be fairly stable. It may be necessary, however, to repeat the procedure of revival several times more before a completely stable culture virus is obtained.

The original culture strain (Chart 1) was carried for more than 2 years without being revived by passage through rabbits. The 88th culture passage produced little or no reaction in the skin of a rabbit yet produced typical vaccinal reactions in the skin of 2 children. It seemed of interest to find out if this culture could be revived by passages through rabbits.

1 cc. of the pooled cultures of the 88th passage (original strain) was injected into each testicle of a rabbit. The culture virus was also rubbed into the scarified skin and injected intradermally. Only a slight papule appeared at the point of intradermal inoculation and no definite vaccinal lesions were seen in the area of scarification during the 7 day period of observation. The animal had no fever. Nevertheless, the testicles were removed on the 4th day after inoculation and appeared practically normal. An emulsion of the testicles was made and 1 cc. of the emulsion was injected into each testicle of another rabbit. Dermal and intradermal inoculations were also made. The testicles were removed on the 4th day and seemed slightly injected. At the point of intradermal inoculation a lesion, 1 x 1 cm. in diameter, developed and 2 discrete pocks were seen in the area of dermal inoculation. The testicular virus was passed to a 3rd rabbit that developed no fever. The testicles in this animal had the appearance of being affected by a mild vaccinia. At the site of intradermal inoculation a lesion, 6 x 5 cm. in diameter, developed, and 2 discrete pocks were observed in the scarified skin. The 4th rabbit had fever, inflamed testicles, confluent vaccinal eruption at the site of scarification, and an intradermal titer of the virus of 10^{-3}. The 5th rabbit had fever, inflamed testicles, and an intradermal titer of the virus of 10^{-5}. The 6th

rabbit had fever and the testicles were inflamed. From an emulsion of the testicles of the 6th rabbit, cultures were initiated and have been designated as the 3rd revived strain. The titer of the 1st culture was 10^{-3}, of the 2nd 10^{-6}, of the 4th 10^{-5}, and of the 7th 10^{-4} (Chart 1).

From the facts presented above it appears that the 88th culture passage of the original strain of culture virus caused little or no reaction in rabbits. Upon repeated testicular passages in rabbits, however, the virus gradually regained its pathogenicity for that host.

TABLE I

Summary of Results of Vaccinations in Man with the Original Culture Strain of Virus

No. of patients	Culture passage	Preservation of culture		Results	Revaccination with New York City virus
		Time	Manner		
1	5	1 mo.	Glycerol at +3°C.	+	
	10	2 days	" " +3° "	+	
12	11	11 "	" " +3° "	12+	
1	11	2 wks.	" " +3° "	+	−
1	11	3 "	" " +3° "	+	
1	14	6 mos.	Glycerol at −10°C.	−	+
1	14	19 "	" " −10° "	+	−
1	42	1 hr.	Glycerol at +3°C.	+	−
1	42	1 wk.	" " +3° "	+	
1	42	2 wks.	" " +3° "	+	
1	82	1 mo.	" " +3° "	+	−
1	82	39 days	" " +3° "	+	
1	86	1 hr.	Without glycerol at +3°C.	+	−
1	88	1 "	" " " +3° "	+	
1	88	1 "	" " " +3° "	+	−

Jennerian Prophylaxis in Man by Means of Culture Virus

Before discussing the effect that *in vitro* cultivation has on the activity of vaccine virus in man it seems advisable to present in tabular form the results obtained in individuals vaccinated with culture virus. With the original strain, 25 people have been inoculated (Table I). Of these, only 1 failed to develop a typical vaccinal lesion. Six of the individuals who reacted to the culture virus were revaccinated with the New York City Board of Health virus and were found to be refractory. With the 2nd revived strain (Table II), 77 people have

TABLE II

Summary of Results of Vaccinations in Man with the 2nd Revived Culture Strain of Virus

No. of patients	Culture passage	Preservation of culture		Results	Revaccination with New York City virus
		Time	Manner		
1	27_2	1 mo.	Glycerol at +3°C.	+	
1	33_2	2 wks.	Desiccated	+	
1	36_2	3 "	"	+	
1	42_2	4 days	Glycerol at +3°C.	+	
1	42_2	1 wk.	" " +3° "	+	
1	42_2	11 days	" " +3° "	+	−
1	42_2	1 mo.	" " +3° "	+	−
4	42_2	1 "	" " +3° "	2+ 2−	
5	43_2	2 wks.	" " +3° "	5+	
1	44_2	6 days	Without glycerol at +3°C.	+	
8	44_2	3 wks.	Glycerol at +3°C.	6+ 2−	
11	47_2	2 mos.	" " +3° "	8+ 3−	
1	42_2	1 mo.	" " +3° "	−	
	49_2	4 days	" " +3° "	+	
1	49_2	4 "	" " +3° "	−	
	51_2	1 hr.	Without glycerol at +3°C.	+	
1	49_2	4 days	Glycerol at +3°C.	−	+
	51_2	1 hr.	Without glycerol at +3°C.	−	
2	50_2	1 day	Glycerol at +3°C.	+ 1−	
9	51_2	4 days	" " +3° "	9+	
8	54_2	3 "	" " +3° "	7+ 1−	
19	56_2	3 hrs.	" " +3° "	15+ 4−	

TABLE III

Summary of Results of Vaccinations in Man with the 3rd Revived Culture Strain of Virus

No. of patients	Culture passage	Preservation of culture		Results
		Time	Manner	
16	5_3	4 hrs.	Glycerol at +3°C.	12+ 4−

been vaccinated, 64 of whom developed typical vaccinal lesions Of those who had reactions, 2 were revaccinated with the New York City Board of Health virus and were found to be refractory. With the 3rd revived strain (Table III), 16 individuals have been inoculated and in 12 of them typical vaccinal reactions occurred. Three children who had been vaccinated with the New York City Board of Health virus were found to be refractory to the culture virus. In summary, 118 individuals have been inoculated with the culture virus and in 100 of them the inoculation was followed by a typical vaccinal pustule. Individuals vaccinated with the culture virus were refractory to a standard dermal strain of calf lymph and *vice versa*. All of the inoculations represent primary vaccinations in infants and children. Approximately one-third of the patients were in the Hospital of The Rockefeller Institute for complete observation during the course of the vaccination. The other two-thirds were vaccinated by us in Dr. Schloss' prophylactic clinic at the Cornell Medical Center and were seen only once after inoculation.

Effect of in Vitro Cultivation on the Activity of Vaccine Virus in Man

With virus from the 5th, 10th, and 11th culture passages of the original strain 15 children were vaccinated. A positive result was obtained in each individual. The reactions were similar to those caused by the New York City Board of Health virus. Consequently, no more children were vaccinated with the virus until it had been passed through 42 successive sets of cultures. Then virus from the 42nd, 82nd, 86th, and 88th sets of cultures were tested in man (Table I). In addition to this, virus from cultures of the 2nd and 3rd revived strains (Tables II and III) were employed for the vaccination of a large number of children. Virus from these cultures did not average as high a percentage of positive reactions as that usually obtained with the New York City Board of Health virus. The reactions, however, were milder than those caused by the Board of Health virus; the children had no fever and were in no way upset. In every respect the results obtained with the culture virus were highly satisfactory.

During the course of the observations it was found that with the later generations of culture virus an area of skin larger than that

usually advised had to be scarified in order to insure a positive reaction. Furthermore, it soon became evident that fresh culture virus (Tables I, II, and III) can be used with complete safety. Such is not the case with green calf lymph. Moreover, in view of our experience with the culture virus, we suggest that it be dispensed in cork-stoppered vials containing enough material for 10 vaccinations instead of in capillary tubes containing only sufficient virus for 1 inoculation. This suggestion is made because the culture virus contains a very small amount of particulate matter and it is believed that sooner or later the virus is adsorbed on these particles which in turn tend to adhere to the sides of the capillary tubes. Under these conditions difficulty is encountered in expressing the virus from the tubes and a low percentage of positive reactions is likely to be obtained.

Vaccination by Means of Intradermal Injection of Culture Virus

The results of intradermal vaccinations have been reported by a number of workers. The literature has been fully reviewed in a communication by Roberts (8). In spite of the favorable reports concerning the matter, most physicians have hesitated to use this method because of the fact that very few vaccine virus preparations are entirely free from living bacteria. Inasmuch as we had a bacteria-free virus that caused mild reactions upon dermal inoculation, we decided to see what it would do when injected intradermally into man.

C. B. received intradermally 0.1 cc. of a 1–10 dilution of culture 42_2 that had been preserved for 11 days in 50 per cent neutral glycerol at +3°C. The first signs of a reaction were observed 9 days later and consisted of erythema and a slight amount of induration. The erythema spread and the induration increased for a few days and then gradually disappeared. No pustule formed and no scar was left. The child was not sick or upset and had no fever. Upon revaccination with the New York City Board of Health virus the child was found to be refractory.

R. R. received intradermally 0.1 cc. of a 1–10 dilution of culture 42_2 that had been preserved for 1 month in 50 per cent neutral glycerol at +3°C. The first signs of a reaction were seen on the 7th day after inoculation. The course of events was practically the same as that described for C. B. with the exception that a very small vesicle formed where the needle was inserted in the skin. Upon revaccination the child was found to be refractory.

J. O'B. received intradermally 0.1 cc. of a 1–10 dilution of culture 44_2 that had been preserved without glycerol for 6 days at +3°C. The first signs of a reaction were observed on the 4th day following inoculation. The course of events was the

same as that described for R. R. The small vesicle resulted in a minute scar. Upon revaccination the child was found to be refractory.

H. J. received intradermally 0.1 cc. of undiluted fresh culture 88. On the 4th day after inoculation a red papule was seen. The course of events was similar to that of the other children. No pustule formed and no scar was left. Upon revaccination the child proved to be refractory.

From the facts presented above it appears that intradermal vaccination with virus that has been passed through a number of cultures is safe. The virus can be used, undiluted or diluted 1–10, in 0.1 cc. amounts either in the fresh or preserved state.

Effect of Storage on the Titer of Culture Virus

It was essential to determine how well the culture virus withstands storage. Consequently, lots of the same virus were preserved in different ways and later tested in rabbits for potency.

One lot of virus was mixed with an equal amount of 100 per cent neutral glycerol, another lot with an equal amount of 100 per cent neutral glycerol to which heated normal chick embryo tissue had been added, and still another lot with an equal amount of 100 per cent glycerol to which sufficient glucose had been added to make a 2.5 per cent solution. Then these lots of virus were placed in small cork-stoppered vials. Half of each lot of vials was stored at $-10°C.$, the other half at $+3°C.$ From time to time a vial of each lot was removed from storage and the virus was titered intradermally in rabbits.

The results of the work described above are shown in Table IV and indicate that the titer of the culture virus in storage ($-10°C.$ and $+3°C.$) gradually fell from 10^{-6} but was still 10^{-4} at the end of a year. Furthermore, virus in a vial that had been stored at $-10°C.$ for 19 months produced a typical vaccinal lesion in a child.

Desiccation of the Culture Virus

Vaccine virus does not maintain its activity well in the absence of refrigeration. It has been shown, however, that a number of viruses retain their activity better if they are frozen and then desiccated while in the frozen state. In view of this fact we performed an experiment in which culture virus (36th passage of the 2nd revived strain) was frozen, desiccated, and then stored in sealed tubes at 37°C. Each week for 5 weeks a tube was removed from the incubator, the original

volume in the tube was restored with sterile distilled water, and the resulting virus mixture was titered intradermally in a rabbit. The results are brought together in Table V and show that the dried virus

TABLE IV

Effect of Storage on Titer of Culture Virus (14th Generation of Original Culture Strain)

Time in storage	Stored at −10°C. in cork-stoppered vials			Stored at +3°C. in cork-stoppered vials		
	Equal volume of glycerol added to culture	Equal volume of glycerol added to culture plus extra heated embryo tissue	Equal volume of glycerol added to culture containing 2 5 per cent glucose	Equal volume of glycerol added to culture	Equal volume of glycerol added to culture plus extra heated embryo tissue	Equal volume of glycerol added to culture containing 2.5 per cent glucose
Titer by calculation before storage	500,000		500,000	500,000		500,000
1 day		1,000,000			1,000,000	
1 mo.	100,000	1,000,000	1,000,000	100,000	1,000,000	100,000
3 mos.	1,000,000	1,000,000	1,000,000	100,000	100,000	1,000,000
5 "	100,000	10,000	10,000	10,000	10,000	1,000
1 yr.	10,000	10,000	10,000	10,000	10,000	1,000
19 mos.	E. Ramon +					

TABLE V

Effect of Storage at 37°C. on Desiccated Culture Virus (36th Generation of 2nd Revived Strain)

Time of titration	Intradermal titer in rabbits
Before desiccation	100,000
After desiccation	100,000
After storage for 1 wk. at 37°C	100,000
" " " 2 wks. " 37° "	1,000
" " " 3 " " 37° "	1,000
" " " 4 " " 37° "	1,000
" " " 5 " " 37° "	10

maintained some of its activity for 5 weeks even at 37°C. In addition to this fact we have shown (Table II) that desiccated culture virus restored to its original volume with 25 per cent glycerol produces typical vaccinal lesions in human beings.

TABLE VI

Initiation of New Cultures from Preserved Cultures

Virus	Time and temperature of preservation	Titer when stored	Amount of dilution in starting cultures	Titer of new series of cultures				
				1st	2nd	3rd	4th	5th
5th generation original strain in glycerol	1 mo. at +3°C.	500,000	1:400	1,100,000	1,000,000	1,000,000		
5th generation original strain in glycerol	3 mos. at +3°C.	500,000	1:400	1,000,000	100,000	100,000		
5th generation original strain in glycerol	10 mos. at +3°C.	500,000	1:400	1,000,000	1,000,000			
8th generation original strain in glycerol	22 mos. at +3°C.	5,000,000	1:400	10,000	10,000		100,000	100,000
43rd generation original strain in glycerol	3 mos. at +3°C.	500	1:400	1	10	1,000	10,000	1,000
6th generation original strain without glycerol	22 mos. at −10°C.	500,000	1:20	100,000	1,000,000		100,000	
33rd generation 2nd revived strain desiccated	5 days at +3°C.	10,000	1:100	100,000	100,000			

Initiation of New Cultures from Preserved Cultures

If the cultivation of vaccine virus is to become a practical procedure, it is essential to know whether new cultures can be initiated with virus from preserved cultures. Therefore, numerous attempts have been made to ascertain the facility with which new cultures can be successfully seeded with virus from cultures that have been frozen and desiccated or from cultures preserved with or without glycerol. We have experienced no difficulty in obtaining fresh cultures in this manner. For convenience a few of the results are shown in Table VI.

DISCUSSION

From the results of the work presented in this paper it is obvious that we have had no difficulty in cultivating a dermal strain of vaccine virus for a period of over 2 years in a medium consisting of bits of viable chick embryo tissue (0.1 gm.) suspended in Tyrode's solution (4–5 cc.) in flasks. This medium was chosen because it is the least likely of all media containing living cells to be contaminated with an unknown or an undesirable virus. It is also evident that culture virus has been successfully employed by us for Jennerian prophylaxis in man. It is hoped that our observations will tempt workers in vaccine virus laboratories to try to adapt this or a similar method of preparation of the active agent for general use. In view of the purity of the virus prepared in this manner and since it causes such mild, yet effective reactions in man, it seems possible that much of the objection to vaccination might be overcome and that the rare but occasional postvaccinal encephalitis might be rendered even more rare, or avoided wholly, by its use instead of that of calf lymph.

Attention should be focussed on the fact that repeated cultivation of vaccine virus in the medium used by us gradually reduced the titer of the active agent for the rabbit and also led to an alteration in the type of lesions produced by the virus in that host. Indeed, the virus that had been cultivated for 2 years in the manner described induced little or no reaction in rabbits. Material from these cultures, however, gave rise to typical vaccinal pustules in man. This observation appears to us to be of importance and among other things seems to indicate that a virus of a desired character for human use can be produced by culture methods. In view of the findings presented at

this time, it is believed that the change in the activity of the virus for the rabbit was not due entirely—and perhaps not at all—to a gradual diminution in the amount of virus in successive sets of cultures, but to some alteration in the character of the virus itself.

SUMMARY

A dermal strain of vaccine virus has been passed through 99 successive culture passages. This procedure led to a diminution in the pathogenicity of the active agent for the rabbit. By repeated testicular passages in rabbits, however, the virus regained its pathogenicity for that host. New cultures were initiated with the revived virus. A culture strain of virus that has been twice revived in this manner has remained fairly stable for the rabbit through 60 culture passages and it produces mild, yet effective vaccinal reactions in man.

Virus in early cultures was not attenuated for man, but later cultures of the original strain and cultures of the 2nd and 3rd revived strains produced mild reactions without fever and discomfort to the patients. Intradermal vaccinations with the culture virus are safe and satisfactory.

With the culture virus 118 infants and children have been inoculated and in 100 of them a positive reaction occurred. The culture virus produced a refractory state to a standard dermal strain of calf lymph and *vice versa*.

Culture virus stored in 50 per cent neutral glycerol at −10°C. or at +3°C. maintained a considerable amount of its activity for at least 1 year. Desiccated culture virus sealed in tubes maintained some of its activity when stored at 37°C. for 5 weeks.

Fresh cultures can be initiated without difficulty from desiccated virus or from virus that has been stored with or without glycerol.

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AMINO NITROGEN CHANGES OF THE BLOOD IN NEPHRITIS

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Amino nitrogen and peptide nitrogen content of the blood in nephritic patients have been frequently determined since the introduction of suitable micro methods. Gasometric determinations of amino nitrogen and peptide nitrogen of plasma and red blood cells separately have, however, not yet been done.

That colorimetric analyses of the same blood samples give lower results for amino acid nitrogen than the gasometric method or the formaldehyde titration, and that colorimetric recovery of amino acids added to blood is incomplete, have been reported by Van Slyke and Kirk (18). The use of different analytical methods may accordingly explain the conflicting results reported in the literature. Thus most observers using the colorimetric method of Folin (Berglund (2), Greene, Sandiford and Ross (6), Schmidt (16), Witts (20), Feinblatt and Shapiro (5)) found no deviation from the normal in series of patients with nephritis, including several cases of uremia. Slightly or moderately increased values of amino nitrogen in uremia were reported with the colorimetric method by Wowsi and Gelbird (21), and by Looney (11) in an individual case of mercuric chloride poisoning shortly before death. Extensive studies have been made in recent years in Volhard's clinic by Becher and Herrmann (1) with a modification of Folin's colorimetric technique; increase of free amino nitrogen of whole blood was frequently observed in patients with severe impairment of the kidney function. Only in a single case, however, did the method used indicate values exceeding 10 milligrams per cent.

Most of the work done with the gasometric method is of earlier date. Using various protein precipitants Bock (3), Okada and Hayashi (14), and Desqueyroux (4), all found increased amino nitrogen of the blood

in cases of advanced nephritis. In nephritic toxemias of pregnancy, on the other hand, Losee and Van Slyke (12), and Morse (13) observed no marked deviation from the normal.

Quantitative determination of peptide nitrogen of blood was first made with exact methods by Hiller and Van Slyke in 1922 (7) by gasometric determination of blood filtrates before and after hydrolysis with hydrochloric acid. A similar procedure of acid hydrolysis has been used by different authors in studies of the peptide nitrogen in nephritis, but in most cases other methods were substituted for the gasometric to determine the amino nitrogen before and after hydrolysis. When comparing results both the method of amino nitrogen determination and the protein precipitant used should be considered. Hülse and Strauss (9), using the formaldehyde titration method of Sørensen, and Hülse and Franke (8), using the gasometric method, observed high values for peptide nitrogen in nephritic patients with hypertension. Jackson, Sherwood and Moore (10), Becher and Herrmann (1), and Schlossmann (15), using the colorimetric method, were, however, unable to find any relation between the blood pressure and the content of peptide nitrogen in the blood in nephritis. A definite increase in peptide nitrogen was frequently seen by Becher and Herrmann (1) in cases of severe renal insufficiency, the peptide nitrogen even occasionally exceeding the concentration of free amino nitrogen; increase of peptide nitrogen with normal amino nitrogen values was sometimes observed.

Methods

For separate analyses of cells and plasma, oxalated blood was centrifuged till constant volume of the red blood cells was obtained; plasma was then syphoned off and the intermediate layer of plasma and cells discarded. Samples of red blood cells were measured with a calibrated pipette "to contain."

Urea was transformed into ammonia by addition of a urease solution and phosphate buffer as described by Van Slyke (17). The proteins of plasma or whole blood were then precipitated by the Folin-Wu method. To plasma or whole blood one volume of 10 per cent sodium tungstate and one volume of 2/3 N sulfuric acid were added, and the mixture was diluted with water to 10 times the original volume of the sample. For precipitation of the proteins of the red blood corpuscles two volumes of 10 per cent sodium tungstate and two volumes of 2/3 N sulfuric acid were used for each volume of cells, but the sample was finally, as in precipitation of plasma and whole blood, made up to 10 times its original volume with water.

Five cc. of filtrate, therefore, represented 0.5 cc. of plasma, red blood corpuscles or whole blood. Usually, however, the filtrate from the red blood corpuscles was again diluted with two volumes of distilled water to obtain sufficient material for analysis. Because of the high amino nitrogen content of the red blood cells the dilution did not diminish the amino nitrogen readings enough to interfere with accuracy.

TABLE I

Showing Reproducibility of Measurements

	Milligrams per cent amino nitrogen	
	I	II
Plasma, unhydrolyzed	4.10	4.07
Plasma, hydrolyzed	4.10	4.03
R.B.C., unhydrolyzed	12.85	12.91
R.B.C., hydrolyzed	18.89	18.03

TABLE II

Amino Nitrogen in Whole Blood during Development of Uremic Coma
Case No. 1, M. J. Hospital No. 7855

Date	Amino nitrogen per 100 cc.			Whole blood non-protein nitrogen per 100 cc.	Whole blood urea nitrogen per 100 cc.	Urea clearance	Condition
	Plasma	Cells	Whole blood				
1931	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*per cent of mean normal*	
September 27			18.0	148	118		Uremia
September 28			13.7	150	119	6	Uremia
October 13							
10:00 A.M.			12.3	171	191		Uremia
9:30 P.M.			21.4	189	141		Coma
October 29	28.8	36.7					Coma

The ammonia of the filtrates, formed by the splitting of the urea with urease, was removed by boiling with milk of magnesia and amino nitrogen afterwards determined by the manometric method of Van Slyke (17).

For determination of peptide nitrogen the urea-and-ammonia-free filtrates were heated on a water bath for 24 hours with equal volumes of concentrated hydrochloric acid. After evaporation of the acid in an open dish each residue

TABLE III

Uremic Cases 2, 3, 4, 5 and 6

Subject			Amino nitrogen per 100 cc.				Blood urea nitrogen per 100 cc.	Urea clearance	Condition
Initials and serial number	Hospital number	Date	Plasma		Cells				
			Free	Peptide	Free	Peptide			
			mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*per cent of mean normal*	
		1932							
E.H. No. 2	8166	May 29	23.4	−1.0	12.2	16.5			Uremia
		June 2	37.3	7.0	24.8	18.0		2	Coma
		1932							
J.D. No. 3	8080	June 29	16.8	−0.6	12.4	6.3			Uremia
		July 7	12.3	1.0	18.4	17.0			Uremia
		1931							
E.B. No. 4	7884	November 5	5.9	1.9	13.5	12.0		61	Good
		November 6	6.9	−0.2	15.3				
		December 21	6.5	0.4	13.1	2.9		90	
		1932							
		May 26	6.5		14.1				
		June 1	6.6		12.4				
		August 29						8	
		September 2	21.3	−5.1	12.9	9.5	140		Coma. Died 48 hours later

TABLE III (*Continued*)

Subject			Amino nitrogen per 100 cc.				Blood urea nitrogen per 100 cc	Urea clearance	Condition
Initials and serial number	Hospital number	Date	Plasma		Cells				
			Free	Peptide	Free	Peptide			
			mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*per cent of mean normal*	
		1931							
M.W. No. 5	6473	December 6....	21 80	−2.29	19 24	10 56	163		Coma. Intravenous glucose
		December 10........	22.40	−1.99	16 25	9 85	143	7 6	Still comatose, but condition improved
		December 15.... . .	10.24	92	11 80	8 26			Condition improved
		December 16.....	7 18	1 33	12.90	8 25			Condition improved
		December 17........ .	5 82	1 66	13 94	7 00	151		Condition good
		December 21..........	21 12	12	14 32	8 08	152		Ascites developing
		December 22..... .	7 29	1 27	14 70	7 28	157	7.2	Fever (till December 24)
		December 26.........	10 20	1 20	10 67	7 32			Condition good
		December 29.........	8 19				127		Condition good
		1932							
		January 1......... ...	5 95	10 21	16 54	7.19	126		Condition good
		January 5......... ..	6.93	.33	14 45	13 54	118		Condition good
		January 11...........	11.52	− .17	16 07	9.36	91	8 4	Condition good
		May 1...............	14 22	− .71	15.51	9.27	95	7.1	Condition good
		September 21..........						3 4	
		September 26	28 58	2.42	7.10	9.38	239		Coma
		September 27..........	28.20	−1.58	9 80	2.81	295		Coma

TABLE III (*Concluded*)

Subject			Amino nitrogen per 100 cc.				Blood urea nitrogen per 100 cc.	Urea clearance	Condition
Initials and serial number	Hospital number	Date	Plasma		Cells				
			Free	Peptide	Free	Peptide			
			mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*per cent of mean normal*	
		1931							
M.R. No. 6	7872	November 24	5.57	− .18	12.24	9.28	20	26	Good
		December 3	11.63	−1.62	13.80	5.05	33	22	Good
		1933							
		May 21	6.43				82	7	Good
		June 13	9.06				182		Uremia
		June 14							
		9.30 A.M.	13.76						Semicomatose. Sample taken before i.v. glucose injection
		9.30 P.M.	10.87						Semicomatose. Sample taken before 2nd i.v. glucose injection
		11.00 P.M.	14.72						Semicomatose
		June 15							
		6.45 A.M.	10.20						Semicomatose
		11.45 A.M.	15.46						Moment of death
M.C. No. 7	8392	See Figure 1							

was neutralized with a few drops of a saturated solution of sodium acetate and finally made up to volume (7). It was found unnecessary to subject the hydrolyzed samples again to boiling with milk of magnesia before the amino acid determination, as identical results were obtained with and without such treatment.

For blank analyses a urease solution was prepared, and precipitated, and the filtrate was diluted as described by Van Slyke (17, p. 442), or Peters and Van Slyke (22). The amino nitrogen content of the filtrate was determined before and after hydrolysis, to obtain the *c.* correction. Separate blanks were determined for analyses of plasma and red blood corpuscles.

The normal magnitude and constancy of results obtained with the above procedure is illustrated by Table I. It gives the results from two samples of the same blood which were centrifuged and analysed separately. The sample of plasma filtrate represented 0.5 cc. of plasma, the sample of filtrate of red blood cells 0.167 cc. of corpuscles.

Studies of amino nitrogen and peptide nitrogen of the blood were made in seven uremic patients. (See Tables II and III.)

CLINICAL NOTES ON UREMIC CASES[1]

Case No. 1. Hospital No. 7855. M. J., female, 33 years. As a child had hemorrhagic Bright's disease following tonsillitis. After several years of active symptoms the patient went into a latent stage, but the renal lesion became active again during a pregnancy, necessitating abortion. In the following period of ten years exhibited hypertension and proteinuria. Two weeks before admission vomiting, edema of feet and dyspnea developed. On admission (September 25, 1931) was in uremia.

Case No. 2. Hospital No. 8166. E. H., female, 21 years. Two years before admission had hematuria and hypertension. Visual disturbances, edema of ankles, vomiting and diarrhea had been present in the last weeks. On admisssion (May 28, 1932) was in uremia.

Case No. 3. Hospital No. 8080. J. D., male, 30 years. Ten months before

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[1] The nomenclature followed here for the different types and stages of Bright's disease is in general that used by Van Slyke, Stillman, et al. (19). In addition, to distinguish the different conditions near and in coma, the following terms are used:

Terminal stage with nitrogen retention. Nitrogen retention without clinical symptoms.

Uremia. Nitrogen retention with clinical symptoms, such as nausea and vomiting, but with normal consciousness.

Semi-comatose condition. Consciousness greatly influenced, but patient still responds.

Coma. Patient unconscious and does not respond.

TABLE IV

Amino Nitrogen and Peptide Nitrogen Content of Blood of Normal Individuals and Non-Uremic Nephritic Patients

Subject	Date	Amino nitrogen in plasma		Amino nitrogen in red blood cells		Urea clearance	Diagnosis
		Free	Peptide	Free	Peptide		
		mgm. per 100 cc.	*mgm. per 100 cc*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*per cent of mean normal*	
A.A.	September 27, 1932	5 20	— .54	11.10	6.95		Normal
S.S.	August 7, 1932	6.56	.55	11.09	6.37		Normal
E.K.	December 10, 1931	5.29	.41	18.07	5.01		Normal
Same	January 21, 1932	4.48	— .16	13 14	7.27		
Same	April 15, 1932	5 51	.17	20 51	9.32		
M.S.	September 28, 1932	4 33	— 06	7.99	5.12		Normal
J.M	September 28, 1932	4 93	— .30	10 15	4.85		Normal
W.M.	September 29, 1932	4.14	.19	8.26	3.95		Normal
Hospital Number							
7943	January 4, 1932	5 46	.23	12.44	7.01	78	Hemorrhagic Bright's disease initial
7986	April 7, 1932	5.93	.37	14.54	4 12	30	
7828	November 25, 1931	5 12	— .72	10.21	5 98	109	Same
7872	November 24, 1931	5.57	— 18	12.24	9 28	26	Same
Same	December 3, 1931	11.63	—1.62	13.80	5 05	22	Same
7450	November 19, 1931	4.45	— .15	10 98	6 82	92	Hemorrhagic Bright's disease active
7842	January 6, 1932	4 90	— .32	8.33	6 53	44	
7884	November 5, 1931	5 87	1 86	13 46	11.97	60	Same
Same	November 6, 1931	6.92	— .22	15.29		60	Same
Same	December 21, 1931	6 48	.44	13 09	2.89	90	Same
7938	December 17, 1931	6.49	.19	8.89	9.57	88	Hemorrhagic Bright's disease latent

TABLE IV (*Concluded*)

Subject	Date	Amino nitrogen in plasma		Amino nitrogen in red blood cells		Urea clearance	Diagnosis
		Free	Peptide	Free	Peptide		
		mgm. per 100 cc.	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*per cent of mean normal*	
7923.......	December 2, 1931	7.83	1.01	13.55	5.55	17	Hemorrhagic Bright's disease terminal
Same......	December 10, 1931	7.84	− .23	17.74	6.86	15	
Same......	April 14, 1932	10.45	.33	19.69	10.55	10	Same
7867.......	October 24, 1931	7.83		15.85		19	Same
8101.......	April 23, 1932	5.00	.47	16.40	6.47	18	Myeloma. Bence-Jones' proteinuria
7922.......	January 3, 1932	8.21	−1.47	12.56	5.79	36	Degenerative Bright's disease same (Etiology pregnancy)
7905.......	November 13, 1931	4.90	− .26	13.56		97	
Same......	November 30, 1931	4.05	.16	12.11	6.07	90	Same
7963.......	January 25, 1932	5.58		16.07		14	Arteriosclerotic Bright's disease
7837.......	December 29, 1931	4.08	.01	12.88	5.58	54	Same
8063.......	April 8, 1932	4.71	1.43	13.94	1.94	35	Same
7876.......	November 9, 1932	7.78	− .54	13.69	5.81	61	Hemorrhagic Bright's disease with nephrotic component
Same......	November 10, 1931	6.88		13.55	7.46	61	
Same......	November 24, 1931	8.04	− .57	12.73	8.50	57	Same
Same......	December 3, 1931	5.66	−1.66	10.25	6.07	58	Same

admission developed tonsillitis, proteinuria and hypertension, followed by edema and ascites. In the last days had had severe nose bleedings. On admission (June 28, 1932) was in uremia.

Case No. 4. Hospital No. 7884. E. B., male, 29 years. Nine years before admission had had respiratory infection with hematuria. Recovered into a latent stage with proteinuria as only symptom. In the last six months had had hyper-

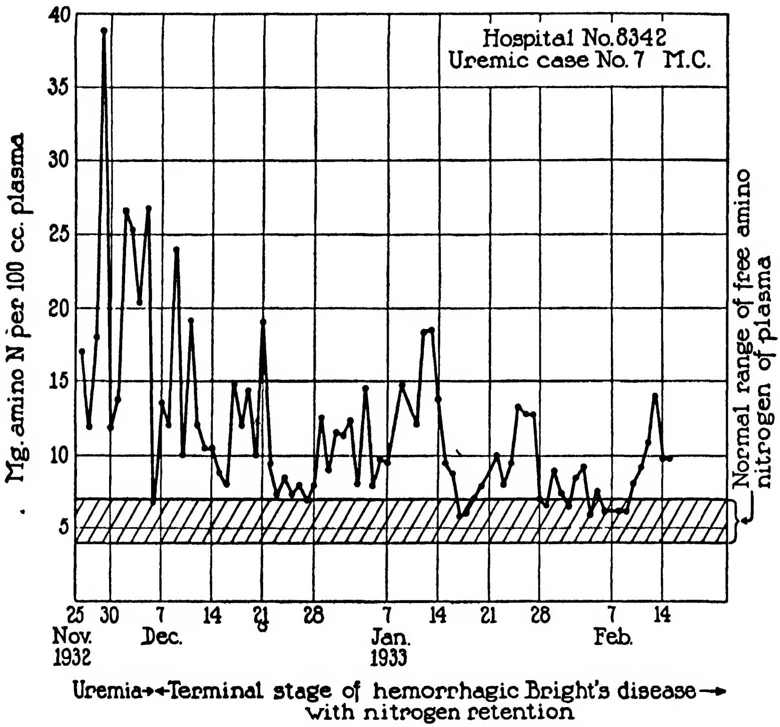

FIG. 1. Amino nitrogen content of blood plasma during and after recovery from acute uremia

tension and severe headache. The patient was observed for several months during the chronic active stage, and during the last weeks of terminal uremia.

Case No. 5. Hospital No. 6473. M. W., male, 15 years. Three and a half years before admission developed generalized edema with proteinuria and reduced kidney function following an attack of upper respiratory infection. In the following period gave evidence of activity in the kidney lesion (microscopic hematuria). Immediately before admission (December 3, 1931) suffered from abnormal sleepiness and attacks of convulsions.

Case No. 6. Hospital No. 7872. M. R., female, 19 years. Four months be-

fore first admission had edema of the legs. On admission (October 21, 1931) macroscopic hematuria, hypertension and reduced kidney function were present. In the following 18 months the nephritis progressed through the chronic active to the terminal stage. Two weeks before last admission (May 4, 1933) reduction of vision and occasional cardiac decompensation were noted. Death occurred on June 15, 1933, after two days of a semicomatose condition.

Case No. 7. Hospital No. 8342. M. C., male, 19 years. Four years before admission developed proteinuria (and hematuria?) following mastoiditis. Went into a latent stage of hemorrhagic Bright's disease. Three weeks before admission had had inflammation of the mandibular region, caused by hemolytic streptococci. In relation to this infection there occurred acute exacerbation of the nephritis with oliguria, gross nitrogen retention and vomiting. On admission (November 25, 1932) was in acute uremia, markedly dehydrated. During the first days in the hospital was at intervals extremely drowsy, but not comatose.

Following treatment of the dehydration the patient made a quick recovery to an excellent general condition. The activity of the renal lesion persisted, however, and the kidney function never exceeded 10 per cent of the average normal urea clearance.

Amino nitrogen determinations of plasma were made daily for a period of about three months. The data are presented in Figure 1. The highest value, 38.7 mgm. of amino nitrogen per 100 cc. of plasma, was observed on November 29, 1932, at a time when the patient was very drowsy.

SUMMARY OF RESULTS

In cases of chronic and acute nephritis with more than 40 per cent of normal urea clearance the plasma amino nitrogen content, determined gasometrically, was found normal, 4 to 6.5 mgm. per 100 cc.

In more advanced cases, but without uremic symptoms, the plasma amino nitrogen was sometimes normal and sometimes increased to 8 to 10 mgm. per 100 cc.

In the pre-coma stage, the plasma amino nitrogen was variable, and might change in 24 hours from practically normal to over 20 mgm. per 100 cc.

In uremic coma, the plasma amino nitrogen was found uniformly high, from 2 to 7-fold normal.

The amino nitrogen fluctuations in the plasma were more marked than in the cells, and more regularly related to the condition of the patients. Significant variations in the plasma peptide nitrogen were not observed.

CONCLUSIONS

Elevation of plasma amino nitrogen tends to be more frequent as renal disease becomes more advanced. The correlation between fall in renal function and rise in plasma amino nitrogen is irregular, however; in some cases the renal clearance approaches the coma level (about 5 per cent of normal clearance) before rise in plasma amino nitrogen occurs. It appears probable, therefore, that the rise in amino acid content is not directly due to renal failure, but to a breakdown in the mechanism for metabolism of the amino acids occurring elsewhere as part of the general debacle.

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THE PRODUCTION IN DOGS OF CHRONIC BLACK TONGUE WITH ANEMIA

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Plates 30 to 32

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Glossitis, stomatitis, and gastrointestinal disturbances are outstanding features of three diseases of human beings: pellagra, sprue, and pernicious anemia. The same triad of symptoms characterizes an acute, spontaneous or experimental disease of dogs, known as black tongue. Experimental evidence indicates that pellagra in human beings and black tongue in dogs can be produced by diets lacking in a particular accessory food factor, and can be successfully treated by supplying that factor. These facts established the symptomatic similarity between the two conditions on the basis of a like etiology. The demonstration by Castle and Rhoads[1] that the same accessory food factor, vitamin B_2 or G, is effective therapeutically in sprue, and by Strauss and Castle[2] that it forms a part of a complex therapeutic agent capable of effecting remissions in pernicious anemia, has suggested that lack of that factor may play a rôle in the etiology of both conditions. Proof of this hypothesis required the production in animals of a disease condition more like sprue and pernicious anemia than was acute black tongue of dogs. If in addition to the oral and intestinal symptoms which are so striking in that disease the features of chronicity and anemia of a characteristic type were added, an experimental syndrome would be established symptomatically like sprue and pernicious anemia in man. Accordingly the production of chronic recurrent black tongue in dogs, with a study of the associated alterations of the blood, was undertaken. The results obtained are presented in this communication.

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A rather comprehensive literature exists concerning black tongue in dogs. In 1907 Chittenden[3] observed that dogs deprived of meat and milk fell ill with the salivation, stomatitis, and diarrhea now recognized as being so characteristic of that condition. The disease picture was similar to one called Stuttgart disease which had been described as occurring spontaneously in Europe. In the southern states of North America it was known as black tongue. In 1917, with Underhill, Chittenden[4] produced a pathological condition marked by salivation, stomatitis, and diarrhea, by feeding dogs a diet composed of boiled peas, cracker meal, and cottonseed oil. It was considered to be similar in all respects to spontaneous black tongue.

The most numerous and detailed experiments dealing with this condition are those of Goldberger and his coworkers.[5–10] These experiments may be epitomized as follows: By the use of a diet composed principally of white corn-meal plus dry peas, casein, cod liver oil, cottonseed oil, and a salt mixture, an acute illness of dogs could be produced in a period of from 4 to 10 weeks. This illness was of brief duration and was marked by stomatitis, salivation, diarrhea, and a fatal outcome. It was very much like the disease produced by previous workers, and was supposed to be experimentally produced black tongue. Untreated animals rarely survived more than a few days. Dermatitis and elevation of temperature were frequently observed. Denton[11] studied the histopathological changes in the tissues of the dogs dying of black tongue in the course of the experiments carried out by Goldberger. The findings were compared with those in human pellagra and were considered to be very similar. From the symptomatic and pathological resemblance, black tongue was considered to be pellagra in dogs. Accordingly a long and detailed series of experiments was performed to ascertain whether the experimental condition in the dog could be prevented and relieved by those foods which appeared to be of prophylactic and therapeutic value in pellagra. A remarkable parallelism was demonstrated.

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[11] Denton, J., *Am. J. Path.*, 1928, **4,** 341.

A study was then made of the factors present in the foods shown to have prophylactic and therapeutic value and which were absent in the diet fed the animals. Investigation of the vitamin content of foods as far as known at that time threw suspicion on that factor known as water-soluble vitamin B. Experiment showed, however, that if materials known to be rich in vitamin B were heated to 120°C. for 1 hour the black tongue-preventing factor was still present. The heat treatment was sufficient to destroy the antineuritic power of the vitamin B complex.

This evidence indicated the presence in the effective material of a new factor named by Goldberger vitamin PP or "pellagra-preventing." This was found to be a factor necessary for the maintenance of growth and the prevention of dermatitis in young rats maintained on an otherwise basal ration. It was variously termed vitamin B_2 or G by other investigators. Yeast was found to be an excellent source of this accessory food substance. Finally, the feeding to human volunteers of a diet somewhat similar to that given the dogs resulted in the production of the symptoms of pellagra.

Wheeler,[12] in reviewing the subject, concluded that black tongue and pellagra were one and the same disease on account of their seasonal and geographical incidence, their common cause and similar course, their identical pathological changes, and their equal response to the same therapeutic and preventive measures.

Shortly after the publications of Goldberger and his colleagues, the subject of the etiology of black tongue was reopened by Underhill and Mendel.[13] They fed to dogs a diet similar to that used in their experiments already discussed. The principal ingredients were cracker meal, peas, and cottonseed oil. An acute disease marked by salivation, stomatitis, and diarrhea was produced, and the therapeutic effect of a variety of materials was tested. They concluded that butter and milk were effective both therapeutically and prophylactically, but that yeast was without potency. Furthermore, they felt that the anti-black tongue effect of butter and milk was subject to a seasonal variation, and that it depended upon the content of carotin. Finally, crystalline carotin was used to supplement the diet fed, and was shown to be effective in preventing and curing the experimental disease. The diet employed by this group of workers was so simple and so generally deficient that it is most difficult to analyze it in respect to any particular ingredient which might be lacking. The discrepancy between the results obtained by the two groups of investigators has never been satisfactorily explained. Neither group, however, apparently considered the similarity of the experimental disease to sprue and to pernicious anemia, and neither studied the associated blood changes. Furthermore, no animals are described in which a chronic pathological state was induced.

A third hypothesis concerning the etiology of pellagra and black tongue has been advanced by Bliss.[14] In a general survey of the diets eaten in regions where

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[12] Wheeler, G. A., *J. Am. Vet. Med. Assn.*, 1930, **77**, 62.

[13] Underhill, F. P., and Mendel, L. B., *Am. J. Physiol.*, 1927–28, **83**, 589.

[14] Bliss, S., *Science*, 1930, **72**, 577.

pellagra is prevalent, he concludes that they are deficient in iron content. Encouraging results are reported to follow the administration of iron in pellagra and in canine black tongue. From these facts, he evolved the hypothesis that both the canine and the human disease are manifestations of a dietary iron deficiency. In a personal communication, Bliss states that the hypothesis still remains neither proven nor disproven.

Stucky and Brand[15] reported experiments on rats in which symptoms of vitamin B_2 G deficiency appeared in spite of a diet containing an amount of iron sufficient to allow hemoglobin regeneration in young, anemic animals.

Methods

Dogs of good size, and of mongrel breed, largely short haired in type, were employed. The animals were kept under uniform conditions, in individual cages, with bedding of shavings. No special care was taken to avoid coprophagy. The diet was composed of the following ingredients: white corn-meal, 6000 gm.; California black eyed peas, 750 gm.; casein (leached—Casein Company of America), 900 gm.; cod liver oil, 225 cc.; cottonseed oil, 450 cc.; rice polishings, 600 gm.; calcium carbonate, 450 gm.; NaCl, 150 gm.

The corn-meal, peas, and casein were mixed and cooked for 2 hours in a steam cooker. The remaining ingredients were then added and thoroughly mixed. The dogs were fed daily and were allowed to eat as much as they chose.

The animals were weighed at weekly intervals, and blood was taken from the jugular vein in a standard amount of potassium oxalate for routine examinations. Counts were done more frequently when the condition of the animal seemed to warrant. Determinations of the numbers of erythrocytes and leukocytes were made in standard pipettes and counting chambers. The hemoglobin was estimated by the Sahli method with a glass standard. The Sahli tubes were carefully calibrated and checked at frequent intervals by the O_2-combining capacity method of Van Slyke.[16] The mean corpuscular volume was ascertained by the method of Wintrobe.[17] Smears of the blood were made on plain cover-slips and those covered with dried brilliant cresyl blue for the staining of reticulocytes.

In treatment, 200 gm. of raw lean beef were fed daily if the animal would eat. If not, a rice polishings concentrate prepared according to the method described by Evans and Lepovsky[18] was administered by stomach tube. Occasionally a brewers' yeast concentrate (Harris) was given. Successful therapeutic results were so difficult to obtain when the disease was at its height that it was often impossible to confine the treatment to some simple material.

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[15] Stucky, C. J., and Brand, E., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 1404.

[16] Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Volume II, Methods, Baltimore, The Williams & Wilkins Co., 1932, 337.

[17] Wintrobe, M. M., *J. Lab. and Clin. Med.*, 1931–32, **17**, 899.

[18] Evans, H. M., and Lepovsky, S., *J. Nutrition*, 1931, **3**, 353.

Prints of the dorsal surface of the tongue were made by applying a thin layer of ink with an ordinary library ink pad. Squares of soft paper were then lightly applied to the lingual surface with a rolling motion. With a little practice, excellent prints could be obtained. In the dogs a general anesthetic was usually employed.

General Results

Early experiments based on the feeding of the diet described, without the addition of rice polishings, were unsatisfactory since death usually occurred before blood changes could be demonstrated. Even with the addition of rice polishings, a substance extremely rich in vitamin B_1 and containing a certain amount of vitamin B_2 G (Evans and Lepovsky[18]), the incidence of acute, fatal disease was high. There appeared to be a striking variation in the ability of the individual animal to maintain life on the diet.

In a certain number of instances, however, a chronic disease developed in which well defined anemia was a feature, associated with a characteristic symptomatology. The averages presented in this publication are based on the findings in 10 dogs. Animals dying of intercurrent disease or from acute black tongue are not included. For the sake of simplicity only those protocols are presented which are fairly typical of the experimental disease.

In general the diet was well taken. Control animals which were given 4 gm. daily of liver extract (Eli Lilly and Company No. 343) ate no more than the dogs maintained on the unsupplemented diet, and remained in perfect health.

Animals which ate sparingly also failed to develop the disease under investigation, but usually died of intercurrent infections. It seemed clear that whatever symptoms developed were due to the absence of an accessory food factor in the diet and not to lack of salts, metallic elements, particular proteins, or caloric intake.

Symptomatology

Certain symptoms, which have not been described as occurring in ordinary acute black tongue, were manifested by the animals which developed the chronic recurrent disease. These symptoms will be described in detail, since upon their presence depends the similarity of

the experimental condition produced to disease syndromes in human beings.

The stomatitis of acute black tongue appears first as localized irregular patches of injection on the floor of the mouth, and on the labial and buccal mucous membranes. This extends until the entire oral mucous membrane is deeply injected and shows a superficial desquamation. In the dogs which ran a chronic course, the mucous membrane changes tended to be more generalized and less acute in character. About 6 weeks after the experimental diet feeding was begun, occasional irregular patches of injection appeared on the surfaces of the labial and buccal mucous membranes. Instead of progressing rapidly to necrosis, the entire lining of the oral cavity frequently became a deep, dusky red in color, and presented a peculiar and striking dry, velvety, rather granular appearance. This change often persisted for many days or even weeks. From time to time, discrete, yellowish, slightly raised aphthae appeared, some of which regressed while others went on to circumscribed desquamation of the epithelium.

Glossitis was a marked feature and was strikingly similar to that seen in sprue and pernicious anemia in human beings. In acute black tongue, the glossitis appears late, is usually confined to the margins of the tongue and is quite extensive, often being almost gangrenous in nature. In the chronic recurrent conditions under discussion, however, the glossitis was frequently widespread, milder in degree, and at times completely unassociated with stomatitis. The first manifestation was a loss of papillae along the margins of the tongue with a faint, smooth, pink to red coloration (Figs. 1 to 3). The involved area was glossy, smooth, and glistening. As the condition progressed, the color became a deeper red, and the area involved in the atrophic process more extensive. Finally, a large part of the dorsum of the tongue might be a deep, fiery red color with complete loss of papillae. Changes in the atrophic areas took place with astonishing rapidity, particularly as healing set in. Large areas of new papillae frequently appeared overnight, and there was a well defined impression that the depth of color might vary from hour to hour. Exactly this same phenomenon has been observed in cases of human sprue. Figs. 4 to 7 show prints taken from the tongues of two dogs at the height of the glossitis and again after regeneration of papillae had taken place. Fig. 8 is a photograph of a tongue presenting advanced atrophic glossitis.

The manifestations of disordered function of the gastrointestinal tract were two: anorexia and diarrhea. In the animals which ran a chronic recurrent course, the appetite was in general well maintained. Only when the salivation and stomatitis were at their height was the diet refused entirely. From time to time, however, periods of several days would elapse during which little food was taken. The diarrhea was variable in character. During an acute exacerbation, it was usually profuse, watery, and brown in color. Blood was frequently passed. In animals running a more protracted course, periods of several days during which the stools

were semisolid to liquid, yellow in color, and voluminous would alternate with periods in which normal stools were passed. Cultures of the diarrheal stools on Sabouraud's medium gave growths of yeast-like organisms in about 10 per cent of the attempts. Control animals never showed such organisms. No attempt was made to identify and classify the type of yeast obtained.

Loss of weight was a striking feature. This again is rarely encountered in studying acute black tongue. The average loss was about 20 per cent of the original weight. That this loss was due to some specific deficit in the diet is shown, as previously mentioned, by the fact that control animals eating the same diet supplemented by 4 gm. daily of liver extract No. 343 maintained their original weight throughout the experiment.

The Anemia

The average maximum variation in blood values observed in the dogs reported are presented in Table I. During the control period

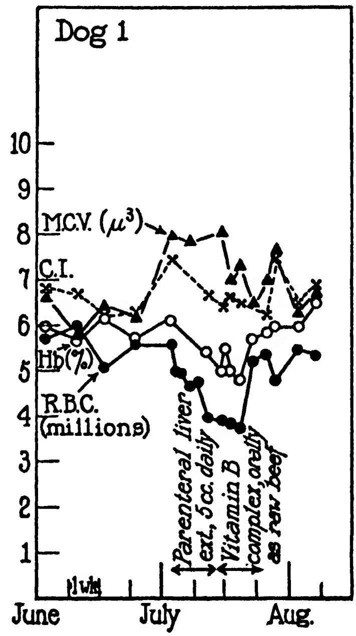
TABLE I

The Average Maximum Variations in Blood Values of 10 Dogs with Chronic Black Tongue and Anemia

	Normal	Low point of anemia	Difference	Per cent difference
Average red cell count	6,440,000	3,100,000	3,340,000	53
" hemoglobin, *per cent*	87.7	56.4	31.3	35.6
" color index	0.685	0.91	22.5	34.5
" mean corpuscular volume, *cubic microns*	60.2	83.1	22.9	33.6

before the diet feeding was begun, the average red cell count was 6,440,000 and the average hemoglobin level, 87.7 per cent. At the low point of the anemia, the average erythrocyte count was 3,100,000, and the average hemoglobin level, 56.4 per cent. There was an average decrease in erythrocytes of 3,340,000, and in hemoglobin of 31.3 per cent. There was in addition an average maximum increase in color index of 22.5 points, and in mean corpuscular volume of 22.9 cubic microns. From the figures it is apparent that the average maximum decrease in numbers of erythrocytes was greater than the average decrease in hemoglobin and was associated with an increase in both color index and mean corpuscular volume. These findings are characteristic of the macrocytic anemias of sprue and pernicious

anemia in man. It should be remembered that the variations in blood values discussed were maximum and that they were not present throughout the period during which the animals were under observation. There was a striking tendency to spontaneous remissions and exacerbations of the anemia. There was at no time a marked increase in the pigment content of the plasma. In a few cases in which blood and plasma volume determinations were done by the vital red method of Rowntree no considerable variations were encountered. Fluctuations in the numbers of leukocytes will be described in another communication. Examinations of the blood of control animals kept under similar conditions failed to show significant variations from normal levels.



TEXT-FIG. 1

PROTOCOLS

Dog 1.—Terrier-hound cross. Text-fig. 1. 3–22–32. Weight 10.3 kilos. R. B. C. 5,540,000. Experimental diet feeding begun. Gastric analysis showed free hydrochloric acid in all samples. 4–21–32. Weight 10.9 kilos. R. B. C. 5,500,000. Salivation was present with mild generalized injection of buccal

mucosa, anorexia, and atrophy with redness of the lateral borders of the tongue. Copious watery diarrhea was present. 400 gm. of meat were fed. 4-25-32. Weight 10.3 kilos. R. B. C. 5,930,000. Recovery followed. 5-21-32. Weight 11 kilos. R. B. C. 4,700,000. Diarrhea, salivation, and mild generalized injection of the buccal mucous membrane were present. The stools were voluminous and yellow in color. Fluids were given intravenously and intraperitoneally with 100 cc. of alcoholic extract of rice polishings. Improvement occurred in 4 days. 6-7-32. Weight 10 kilos. R. B. C. 5,000,000. Active stomatitis with the formation of a thin necrotic membrane was present. Salivation and diarrhea were copious, with marked redness and atrophy of the lateral borders of the tongue. Physiological saline solution was administered intravenously and intraperitoneally. Rice polishings concentrate was given orally. Improvement followed in 4 days. 7-7-32. Weight 8.7 kilos. R. B. C. 4,900,000. Fourth attack, with marked, generalized stomatitis, great salivation, and active, yellow diarrhea. Marked atrophy of the tongue was present. The administration of physiological saline solution intravenously, and of rice polishings extract orally, was followed by improvement in 6 days.

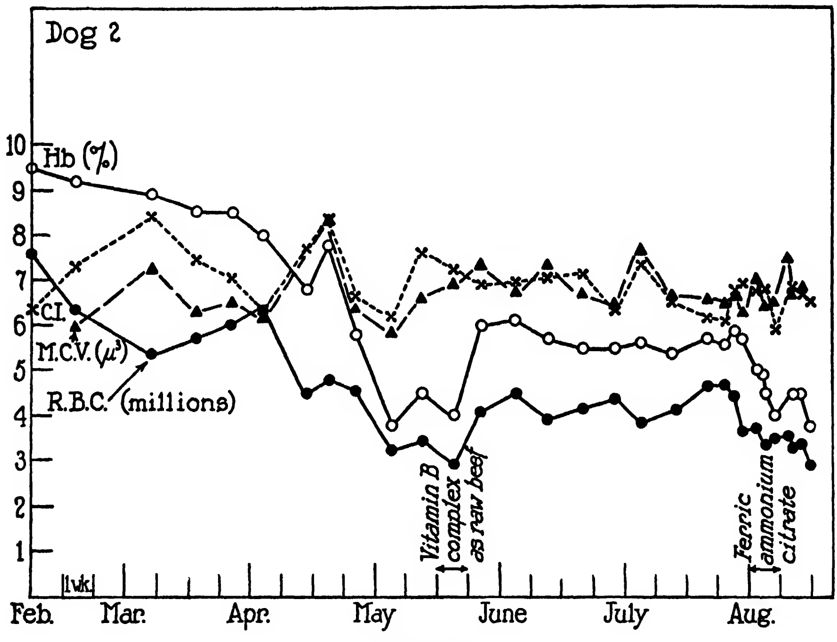
After this attack there was a sharp fall in the blood values to a low erythrocyte count of 3,740,000 cells per c.mm. The color index was somewhat greater than normal, and there was a slight increase in the mean corpuscular volume. The course of the blood changes is shown in Text-fig. 1. Liver extract (Lilly No. 343) prepared for parenteral injection was administered intramuscularly each day for 10 days without improvement in blood values.

7-18-32. 100 gm. of raw beef were fed daily in addition to the experimental ration. There was a prompt return of the blood values to a high level.

This animal had four acute attacks of stomatitis and gastrointestinal disturbance. Following the last attack anemia developed. No improvement in blood values followed the parenteral injection of liver extract but striking improvement followed meat feeding. In the interim between acute attacks, the buccal mucous membrane of this animal presented a dry, irregular, deep red, granular appearance. A variable degree of lingual atrophy was constantly present with exacerbations during acute attacks. No decrease in the ability of the stomach to secrete hydrochloric acid was observed.

Dog 2.—Great Dane-bull cross. Text-fig. 2. 2-6-32. Weight 20.5 kilos. R. B. C. 7,500,000. The experimental diet feeding was begun. Gastric analysis showed free hydrochloric acid and ferments in all samples. 3-7-32. Weight 20.7 kilos. The animal was inactive and salivating mildly. Patchy reddening of the mucous membrane of the upper lip was present. 3-8-32. Weight 20 kilos. R. B. C. 5,300,000. Extensive uniform injection of the buccal and

labial mucous membrane had developed, and salivation was marked. Diarrhea was active with liquid, brown, watery stools. 3–12–32. Weight 20 kilos. R. B. C. 5,700,000. A general diffuse redness of the buccal mucous membrane was present with a rather dry, granular appearance. Nearly all the stools after 3–7–32 were watery in consistence and ranged from brown to yellow in color. This condition persisted until 3–24–32. 3–24–32. Weight 18.9 kilos. R. B. C. 6,000,000. The lateral margins of the tongue showed marked atrophy of papillae with a striking purplish red color. An illustration of the tongue at this stage is shown in Figs. 3 and 8. The mouth and tongue changed very little until 4–2–32. The diar-



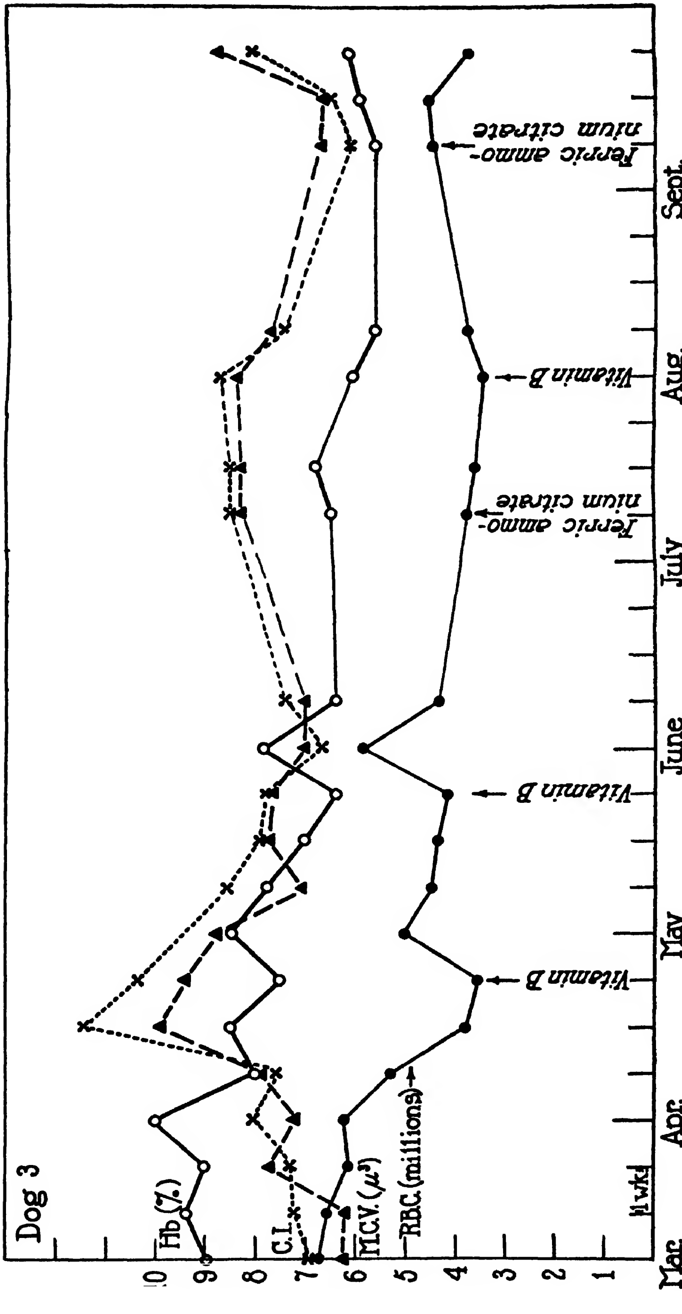
TEXT-FIG. 2

rhea persisted. 4–2–32. Weight 18.9 kilos. R. B. C. 4,490,000. The margins and tip of the tongue had become glass-smooth and a fiery red in color. A print of the tongue at this stage is shown in Fig. 5. There was the faintest trace of injection of the buccal mucous membrane. The dog was eating the diet well and was in good condition. 4–5–32. Weight 18 kilos. R. B. C. 4,400,000. The fiery red color of the tongue was rapidly fading, and new papillae were appearing in the areas which had been completely atrophic (Fig. 4) Otherwise the mouth showed no abnormality. Loose voluminous yellow stools were the rule, but occasional brown, formed stools were passed. 4–12–32. Weight 17 kilos. R. B. C. 4,790,000. In the interim there was little change. On this date the margins

of the tongue were once more smooth, atrophic, and a fiery red in color. The stools were large, yellow, and semifluid to fluid. From these stools a yeast-like organism was grown on Sabouraud's agar. The animal was very weak and listless. 4–20–32. Weight 15 kilos. R. B. C. 4,500,000. The bright red injection of the lingual borders had faded, and new papillae were appearing. The diarrhea was much less but still present intermittently. 200 gm. of raw, lean beef were added to the diet daily for 10 days. Within a few days the lingual inflammation and atrophy had disappeared, the diarrhea had ceased, and the animal gained rapidly in health and strength. There was an improvement in the blood levels. From this point on there was a steady fall in the number of erythrocytes to a low level of just under 3,000,000. The changes in the other values are graphically shown in Text-fig. 2. 7–5–32. Weight 14 kilos. R. B. C. 3,600,000. The tongue was once more atrophic, and presented a bright red coloration of the margins. Diarrhea was intermittent. The stools were bulky, yellow, and fluid to semifluid in consistence. Ferric ammonium citrate, 1 gm. daily, was added to the diet. The condition of the tongue and gastrointestinal tract persisted unchanged to 8–10–32, when the animal was found dead. The pathological changes will be described elsewhere.

This animal showed chronic, recurrent, atrophic glossitis and gastrointestinal disturbance for a period of 6 months. As seen from Text-fig. 2, there was an anemia of advanced degree which was improved by feeding meat, and did not respond to iron administration. The pronounced, necrotic mucous membrane lesions so commonly seen in acute black tongue were not encountered in this instance. The extent and marked character of the lingual lesions may be seen in the tongue print, Fig. 5, the photograph, Fig. 8, and in the colored Fig. 3. There was a marked loss of weight. Despite the striking gastrointestinal symptomatology, there was at no time a decrease or loss in the ability of the stomach to secrete free hydrochloric acid. The bone marrow at autopsy was extremely red and hyperplastic.

Dog 3.—Black and tan terrier. Text-fig. 3. 3–9–32. Weight 16.1 kilos. R. B. C. 6,700,000. Experimental diet feeding begun. Gastric analysis showed free hydrochloric acid in all specimens. 4–20–32. Weight 14.8 kilos. R. B. C. 3,300,000. The food was refused, and pronounced, watery diarrhea was present. Multiple red patches were present on the buccal mucous membrane. The dog was fed 200 gm. of raw beef daily for 5 days, when the mouth became free from lesions, and the appetite returned. There was an improvement in the blood values. 5–27–32. Weight 15.1 kilos. R. B. C. 4,440,000. Copious, watery diarrhea was present. A mild, patchy injection of the buccal mucosa had appeared. 5–28–32. Weight 15.6 kilos. R. B. C. 4,400,000. Marked redness and atrophy of the

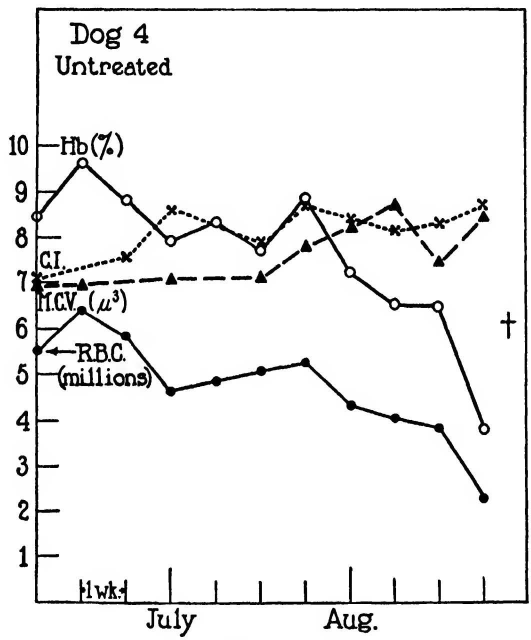

Dog 3
Hb (%)
C.I.
M.C.V. (μ³)
R.B.C. (millions)
1 wk.
Vitamin B
Vitamin B
Ferric ammonium citrate
Vitamin B
Ferric ammonium citrate
Mar.
Apr.
May
June
July
Aug.
Sept.
10
9
8
7
6
5
4
3
2
1

TEXT-FIG. 3

tongue margins had developed and the papillae had entirely disappeared (Figs. 1, 2, and 7). A mild diarrhea with bulky yellow stools was present. The dog was inactive. This condition continued for 4 weeks. There were variations in the intensity of the atrophic glossitis, but at no time did more than a few papillae return. Cultures of the stools on Sabouraud's medium were repeatedly positive for yeast-like organisms Meat, 200 gm. daily, was fed for 4 days, with temporary improvement in blood levels. 6–23–32. Weight 14.2 kilos. R. B. C. 3,800,000. The tongue atrophy had now begun to disappear, and many new papillae were present (Fig. 6). The buccal mucous membrane presented a dry, granular appearance. Except for persistent diarrhea the animal was in good condition and eating well. 7–5–32. Weight 12.6 kilos. R. B. C. 3,800,000. In the interim there was little change. Loose, yellow stools containing yeast were passed nearly every day. The tip and borders of the tongue were smooth and a deep pink in color. Ferric ammonium citrate was administered, 1 gm. daily, for 2 weeks without improvement in blood values, glossitis, or gastrointestinal symptoms. There was a slow but constant increase in the activity of the symptoms. 7–30–32. Weight 11 kilos. R. B. C. 3,500,000. The animal appeared unusually quiet. The glossitis was marked. 200 gm. of raw beef were fed daily for 6 days followed by striking improvement. The redness of the tongue faded and papillae reappeared. The diarrhea ceased for a few days. 8–14–32. Weight 10.7 kilos. R. B. C. 3,700,000. The diarrhea began once more, and mild atrophic glossitis developed. The symptoms continued until 9–24–32 when meat was again fed and the symptoms disappeared. There was a rise in blood values. 10–10–32. Weight 14 kilos. R. B. C. 5,850,000. The buccal mucosa again took on the red, dry, granular appearance described previously. An intermittent diarrhea with bulky yellow stools began. The tongue was very red and the papillae were completely absent on the tip and borders. Ferric ammonium citrate, 1 gm. daily, was administered for 10 days without effect on either symptoms or blood. 10–16–32. Weight 16 kilos. R. B. C. 5,200,000. The buccal mucous membrane was very injected, and a large number of yellow aphthae were present. Atrophic glossitis was clear-cut. The diarrhea was active, and the stools contained blood. 10–26–32. Death occurred.

This animal, like Dog 2, ran a prolonged, chronic course with pronounced glossitis, persistent diarrhea, and a marked anemia. As may be seen from Text-fig. 3, the blood showed cycles of macrocytosis relieved by meat feeding. No effect of liver extract (Lilly No. 343) injected intramuscularly was observed, nor was iron in large doses potent in relieving the symptoms or in bringing about improvement in blood values. At no time was any decrease in the hydrochloric acid content of the stomach noted. The femoral bone marrow was extremely red and hyperplastic. There was a maximum weight loss of 5.4 kilos.

Dog 4.—Short haired shepherd. Text-fig. 4. 6–14–32. Weight 17 kilos. R. B. C. 6,400,000. Experimental feeding was begun. Gastric analysis showed free hydrochloric acid in all samples. The diet was well taken, and no abnormalities of the mouth were noted for 8 weeks. As shown in Text-fig. 4, there was a progressive pronounced drop in the blood values, and a steadily increasing macrocytosis. A dry, injected, oral mucous membrane was constantly present. 9–2–32. Weight 14 kilos. R. B. C. 2,260,000. The tip and lateral margins of the tongue were completely atrophic and a brilliant scarlet in color. Diarrhea was present. Liver extract No. 343 was injected intravenously and intramuscularly with large



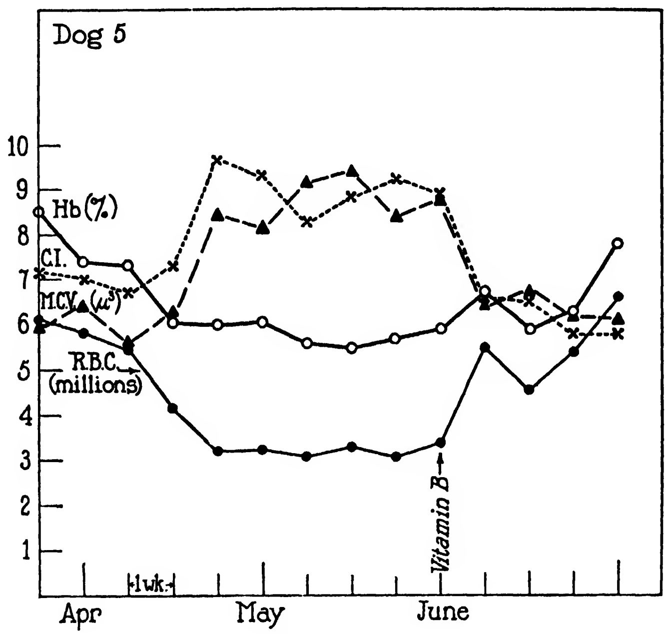
TEXT-FIG. 4

amounts of physiological saline solution. 9–3–32. The animal was prostrate. The therapy was repeated, but death ensued on the following day.

This animal, in contrast to the three previously described, showed a maximum of hematological effect and only terminal lingual and gastrointestinal symptoms. This difference in the clinical course must be ascribed to individual variations in the experimental animal. No treatment was administered until late in the disease. Comparison of the chart of this animal's course with those of the treated dogs shows

strikingly the effect of early therapy with material rich in vitamin B_2 G on the experimental disease.

There was a maximum weight loss of 3.6 kilos. The bone marrow at autopsy was not red and hyperplastic in this instance, a fact in keeping with the short course and rapid fall in blood values. There was no decrease in the ability of the stomach to secrete hydrochloric acid.

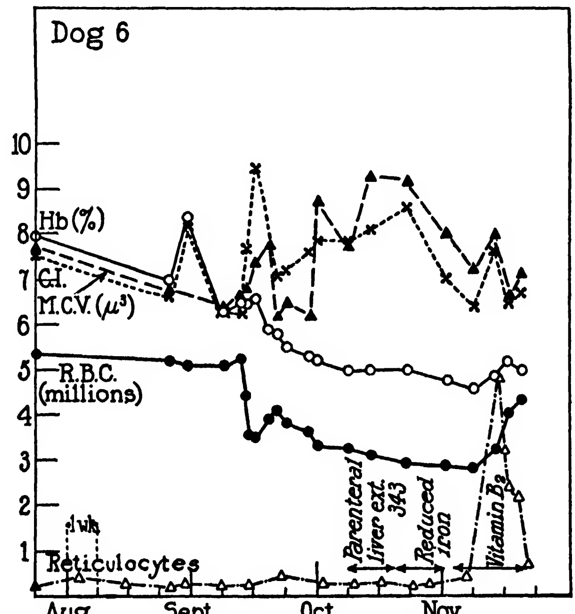


TEXT-FIG. 5

Dog 5.—Black shepherd. Text-fig. 5. 1–17–32. Weight 17.9 kilos. R. B. C. 6,000,000. Experimental diet feeding begun. Free hydrochloric acid was present in all specimens of gastric contents. 3–11–32. Weight 10.2 kilos. R. B. C. 4,100,000. A mild, patchy injection of the buccal mucous membrane was present. A slow spread of the lesions took place until 3–22–32 when they disappeared spontaneously. 3–29–32. Weight 16.4 kilos. R. B. C. 3,200,000. The macrocytic anemia continued to be present. A few patches of redness remained in the mouth, and the dog was rather inactive. An intermittent diarrhea had set in with soft, bulky, yellow stools. 4–24–32. Weight 14.8 kilos. R. B. C. 3,100,000. In the interim there were a persistent diarrhea and generalized velvety injection of the buccal mucous membrane. Atrophy of the tongue margins appeared and

increased in intensity. The animal was inactive. Cultures of stools on Sabouraud's agar were repeatedly positive for yeast-like organisms. Autolyzed yeast, 30 gm. daily, was given by mouth followed by a prompt amelioration of symptoms and improvement in blood values.

Although this dog was fed the experimental diet for a long period after the improvement in blood values shown in the chart, no further anemia developed. Death in an attack of acute black tongue terminated the experiment. The only possible explanation of this se-



TEXT-FIG. 6

quence of events in the light of present knowledge is that a variable individual factor predisposing to anemia is required. This occasional relative independence of oral symptoms and anemia is not uncommonly encountered in dealing with sprue and pernicious anemia in human beings. There was a maximum weight loss of 4 kilos. No decrease in the acidity of the gastric contents was noted.

Dog 6.—Mongrel terrier. Text-fig. 6. 7–26–32. Weight 15 kilos. R. B. C. 5,380,000. The experimental diet feeding was begun. 9–3–32. Not weighed.

A diffuse redness of the buccal mucous membrane was observed for the first time. Bulky, semisolid to liquid, yellow stools were passed almost daily. 9–15–32. Weight 12.6 kilos. R. B. C. 3,500,000. Profuse yellowish diarrhea was present daily in the interim. The tongue showed a very striking redness and marginal atrophy. A mild generalized injection of the buccal mucosa was present. Stool cultures were repeatedly positive for yeast-like organisms. 10–8–32. Weight 11.8 kilos. R. B. C. 3,100,000. Some papillae had reappeared on the tongue and the redness had largely faded. The diarrhea decreased. Liver extract No. 343 (Lilly) was injected daily in 5 cc. amounts. As seen from the chart no improvement in blood values resulted. 10–18–32. Weight 11.2 kilos. R. B. C. 2,900,000. The atrophic glossitis and gastrointestinal manifestations were improved. The administration of reduced iron, 1 gm. daily, for 10 days was begun. 10–28–32. Weight 11.0 kilos. R. B. C. 2,900,000. No improvement of blood values had occurred, and iron therapy was discontinued. Atrophy and redness of the tongue borders were present with a moderate injection of the buccal mucous membrane. 11–1–32. Weight 10.5 kilos. R. B. C. 3,280,000. Autoclaved brewers' yeast autolysate (Harris) was given, 15 gm. daily for 10 days. A mild reticulocyte rise and improvement in blood values resulted in an erythrocyte count of 4,370,000.

After the routine feeding of the experimental diet was reinstated, this animal died in an acute attack of black tongue without marked anemia.

The course of this animal's illness is particularly important since three methods of therapeusis were employed, and all of the clinical manifestations under discussion were present. The lingual atrophy and redness were striking and present nearly constantly for a long period. The diarrhea was yellow, voluminous, and persistent. Cultures on Sabouraud's agar repeatedly were positive for yeast. There was a macrocytic anemia of marked degree which failed to improve upon the administration of iron or of injected liver extract No. 343 (Lilly). A reticulocyte rise, a drop in color index and mean cell volume, and a rise in erythrocyte numbers and hemoglobin content occurred when material rich in vitamin B_2 was fed. During the time the animal was under observation, there was a loss of weight of 4.65 kilos.

DISCUSSION

From the findings presented, it appears that a chronic recurrent disease in dogs was produced by the feeding of a particular diet. The disease was marked by chronic stomatitis with acute exacerbations, by salivation, by prolonged and marked atrophic glossitis, loss

of weight, diarrhea, and, most important, anemia. The anemia was moderately severe, bore a relationship to the duration and intensity of the concurrent symptomatology, and was at times strikingly macrocytic in character. Certain factors in this experimental disease deserve special consideration.

The exact etiology of the condition produced still remains obscure. The diet fed was selected because it could be relied upon to produce the cardinal oral symptoms desired. It was supplemented with rice polishings to rule out the possibility that a deficiency in antineuritic vitamin B_1 was present. This supplement undoubtedly contained a small amount of vitamin B_2 G. The prophylactic and therapeutic effect of materials rich in vitamin B_2 G, such as meat, rice polishings concentrate, and autoclaved yeast, was striking. These facts make a strong argument for the hypothesis that vitamin B_2 G or some closely related substance was the missing factor in the diet. That vitamin can be employed only in an extremely crude form. Titration of the diet fed for its power to cause growth in young rats has not yet been carried out; hence an even approximately exact statement as to the absent factor cannot be made. It seems clear that lack in vitamin A or antineuritic B_1 was not at fault. The same diet supplemented with liver extract powder (Lilly No. 343), 4 gm. daily, maintained the animals in perfect health. This material is almost free of salts and of protein (Cohn[19]). It is rich in vitamin B complex (Guha[20]). Hence it appears that the diet contained no material which was toxic *per se* and was not deficient in particular proteins or in mineral constituents. Carotin in large doses parenterally administered was not therapeutically effective nor was iron ammonium citrate. Lack of these two materials cannot be considered causative. Pending further experimentation, lack of some substance closely associated with vitamin B_2 G seems to be the most probable etiology.

Variability in the response of dogs kept under the same experimental conditions was striking. No explanation of this fact is at hand other than a constitutional variation in the host. A similar lack of uniformity is found in the human response to particular dietary de-

[19] Cohn, E. J., McMeekin, T. L., and Minot, G. R., *J. Biol. Chem.*, 1930, **87**, p. xlix.

[20] Guha, B. C., *Lancet*, 1931, **1**, 864.

ficiencies. Most of the animals eventually died in an attack of acute black tongue even when the diet was supplemented with rice polishings. Great patience was required to obtain a convincing series of animals presenting a more or less chronic disease.

The criticism may well be advanced that the changes in cell volume reported might have been the result of slight changes in tonicity of the blood plasma, themselves the result of dehydration due to diarrhea. That possibility seems unlikely since no consistent correlation between diarrhea and change in cell size was observed. Indeed, the presence of frequent liquid stools was usually associated with a rise in the blood count and a decrease in the average cell size. Furthermore, loss of water from the intestinal tract should result in an increased tonicity of plasma and a decrease in cell size, rather than the reverse.

At the outset of the experiment, it was thought possible that a loss of the ability of the stomach to secrete hydrochloric acid would be encountered. This only occurred rarely, as a terminal event, and will be described elsewhere. Indeed, those animals which ran the most chronic course were those which maintained their gastric secretory function.

The changes in the bone marrow of the animals autopsied at the height of the anemia were frequently striking and characteristic. The histological details will be presented in a subsequent communication. It suffices to state here that the femoral marrow was the site of intense cellular activity with nearly complete absence of fat and replacement by young hematopoietic cells. In many respects this marrow change was similar to that seen in severe sprue (Rhoads and Castle[21]) and in pernicious anemia (Peabody[22]). There was a change in the extent of active marrow as compared with the femoral marrows of normal dogs and of the experimental dogs upon which biopsies were performed before the feeding of the experimental diet was begun. There was also a change in the cell type of the active marrow. This was best seen by comparing the histology of normal active vertebral marrow which was predominantly normoblastic with the pathologically active femo-

[21] Rhoads, C. P., and Castle, W. B., *Am. J. Path.*, in press.

[22] Peabody, F. W., *Am. J. Path.*, 1926, **2**, 487.

ral marrow of anemic dogs which was predominantly megaloblastic in type.

A striking fact was the failure to demonstrate an improvement in symptoms and blood values following the parenteral administration of large doses of liver extract. Three instances of this fact are included in the individual protocols. Twelve other trials of parenteral liver extract as a therapeutic agent were followed by similar failure. Moreover, the daily administration of that amount of liver extract derived from 50.0 gm. of whole liver prepared for parenteral administration injected intramuscularly each day failed to prevent the development of anemia and glossitis. That this material is specific in relieving the symptoms of sprue and pernicious anemia has been conclusively shown. Since the oral administration of substances rich in vitamin B_2 G did produce improvement in blood values, it would appear that the dog utilizes vitamin B as such in hematopoiesis and not as an intermediate product of gastric digestion as does the human being. Further experiments on this point are in progress.

No consideration of anemia in the dog should fail to include the possibility of infection with *Bartonella canis* (Kikuth[23]). Examination of blood smears from the animals included in this report failed to reveal *Bartonella* bodies. That an infection with the organism may be latent and still depress the blood values under abnormal conditions is quite possible. It can only be stated that in the experiments no evidence of such an infection was found.

SUMMARY AND CONCLUSIONS

1. By the feeding of a particular diet, apparently lacking a substance closely associated with vitamin B_2 G, a chronic disease may be produced irregularly in dogs.

2. The disease is characterized by atrophic glossitis, diarrhea, loss of weight, and anemia.

3. The disease can be prevented and relieved by materials rich in vitamin B_2 G.

[23] Kikuth, W., *Centr. Bakt., 1. Abt., Orig.*, 1929, **113**, 1.

EXPLANATION OF PLATES

Plate 30

Figs. 1 to 3. Paintings of the tongues of dogs with chronic black tongue showing various degrees of injection and atrophy of papillae.

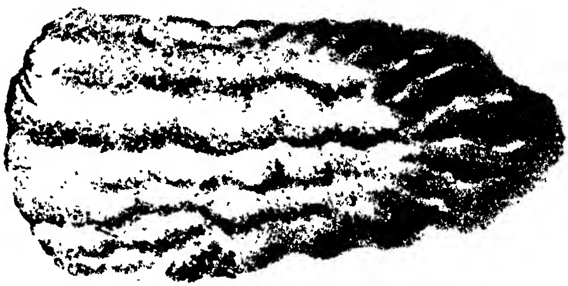
Plate 31

Figs. 5 and 7. Prints taken from the dorsal surfaces of the tongues of dogs with the atrophic glossitis of chronic black tongue.

Figs. 4 and 6. Prints from the same tongues after recovery had taken place.

Plate 32

Fig. 8. Photograph of the tongue of a dog with chronic black tongue showing atrophy of the papillae of the lateral lingual borders.



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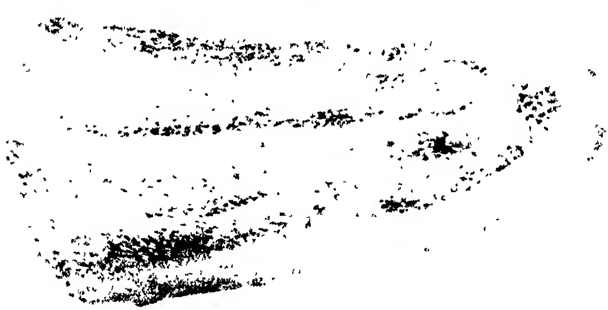

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(Rhoads and Miller: Chronic black tongue with anemia)

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(Rhoads and Miller. Chronic black tongue with anemia)

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Photographed by Louis Schmidt

(Rhoads and Miller: Chronic black tongue with anemia)

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THE EFFECT OF SPLENIC CONTRACTION ON THE FORMED ELEMENTS OF THE BLOOD IN A CASE OF ANEMIA AND SPLENOMEGALY

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The function of the spleen as a reservoir for the formed elements of the blood is a comparatively recent conception. Experimental studies have dealt principally with the number of erythrocytes stored or static in the splenic pulp and have neglected other equally significant cellular elements. Such studies, moreover, have been almost entirely made upon animals and the application of the results to problems of human physiology has not been proven.

Stukeley (1) suggested in 1723 that the spleen manifested relaxation and active muscular contraction. Gray (2) in 1854 mentioned the function of the spleen as that of a "safety-valve" for the storage of blood apart from the general circulation. Roy was the first physiologist to observe the actual rhythmic movements and contraction of the spleen in laboratory animals (3). His work was confirmed in 1896 by Schäfer and Moore who also demonstrated the innervation of the organ (4). The present day views of the function of the spleen as a blood reservoir are largely due to the work of Barcroft and his associates (5). Barcroft brought the spleens of dogs out through a slit in the abdominal wall and left them in this position for considerable periods of time, protected only by saline dressings. With these preparations he demonstrated the rhythmic movements of the spleen and also the active contraction after various stimuli such as heat, exercise, and emotion. It was shown in later experiments that the spleen of the dog contracts to one-half or one-third of its normal size during exercise and to an even greater extent after severe hemorrhage or the death of the animal. His work with carbon monoxide has clearly demonstrated that the spleen serves as a reservoir of red blood corpuscles.

Many studies have been made on the hemoglobin content and the erythrocyte count of blood expelled from the spleen both during active contraction and while the organ was quiescent. Consistent results have not been obtained. In some cases identical values were obtained for blood from the splenic artery and vein. Cruickshank (6), working in Barcroft's laboratory, by cannulizing the inferior mesenteric vein, collected blood during contractions of the spleen. At the same time he measured the contraction of the spleen and the quantity of blood expelled. From experiments of this nature he determined that the blood expelled was often richer in hemoglobin than was the blood of the general circulation. The concentration of hemoglobin varied during the contraction, the maximum value being 20 to 40 per cent higher than that of the normal peripheral blood. The amount of blood expelled from the spleen by a single contraction was estimated to be 2.6 to 5.6 per cent of the total blood volume of the animal.

An opportunity for the detailed study of the spleen as a reservoir of blood was presented to us in a patient in which there was an anemia and splenomegaly. The spleen in this patient was easily demonstrable and could be visualized by the x-ray. It was observed that the spleen invariably contracted following the intravenous injection of liver extract, a therapeutic measure instituted with a view to relieving the anemia. The present paper deals with the changes in the number and character of the formed blood elements which took place during the splenic contractions induced by this and other methods.

Methods

The general plan was to study the character and cellular composition of the peripheral blood before and after the induced contraction of the spleen, and to compare these findings with the changes in the size of the organ, as determined by x-ray.

Blood samples were taken by venipuncture both before and after the splenic contraction. The red and white corpuscles were enumerated in the usual way in these samples. Hemoglobin determinations were made by the Sahli acid hematin method, using calibrated tubes and standards. The mean corpuscular volume of the erythrocytes was determined with the Wintrobe hematocrit tube. Enumeration of the blood platelets was then done on blood obtained from a freely bleeding puncture of the ear lobe. Three per cent sodium citrate solution prepared freshly every day was used as a diluting fluid in the red corpuscle pipette. Great care was exercised to keep the sodium citrate solution and all the glassware

free from dust and dirt. Fragility tests of the red corpuscles were done by the usual method, using sodium chloride solutions in dilutions varying from 0.52 to 0.28 per cent. Controls were made on normal bloods at each determination. Blood volume determinations by the vital red method of Rowntree (7) were made before and after splenic contractions. X-rays of the spleen were taken before the injection and at regular intervals following the contractions. The surface area of the splenic shadow was measured on these x-rays with a Keuffel and Esser planimeter. The upper pole of the spleen could not be definitely outlined; therefore identical points were taken on the ribs from which measurements were made.

Observations

Clinical summary. The patient, a 55 year old Puerto Rican woman, entered the hospital complaining of attacks of diarrhea and abdominal discomfort of four and a half years' duration. A diagnosis of sprue had been made fifteen years ago while the patient was a resident of Puerto Rico. Following her arrival in this country thirteen years ago the diarrhea ceased and the patient gained weight. There was no history of yellow fever, typhoid, or malaria.

The present illness began about four and a half years ago when the patient noticed five or six loose bowel movements a day. These attacks lasted about three weeks and occurred once or twice a year. An abdominal mass was noticed on the left side of her abdomen three years ago. Six months before entry the patient noticed shortness of breath and pallor. Three months later a local doctor found a severe anemia and transfused the patient. Following this the patient improved and was well until one month before entry, when her diarrhea and abdominal discomfort returned. At the same time her pallor was again noticed and she became short of breath on exertion.

Physical examination. The patient was markedly emaciated and had moderate pallor of her skin and mucous membranes. No lymphadenopathy was present. The heart and lungs were normal. The abdomen was moderately distended. A large, hard mass was palpated on the left side of the abdomen. The lower edge of the mass extended to the level of the umbilicus, the medial edge extended to the mid abdominal line. A notch was felt in this medial border. The red blood count was 3,240,000, hemoglobin 71 per cent, white cell count 1,250. The differential counts done with Wright's stained smears and by the supravital technique showed no abnormal cells. The fragility of the red blood cells was within normal limits. Gastric analysis showed no free hydrochloric acid after histamine expression.

For the treatment of the anemia liver extract was given intravenously. During the first injection, which was of twenty minutes' duration, the patient complained of severe abdominal pain, localized over the splenic area. Nausea and vomiting promptly occurred. Toward the end of the injection marked flushing of the face, neck, hands, and arms was present. Immediately following the injection palpation of the abdomen revealed that the spleen, the lower edge of which had been just

below the level of the umbilicus, had contracted in size until its lower edge was palpable about 1.0 cm. below the costal margin. Moreover, whereas the spleen had been hard before injection, it was found to be very soft in consistency after the shrinkage in size. This observation suggested the studies reported in this communication. Table I represents a typical chronological protocol of these studies.

TABLE I

Chronological Protocol

Time	Remarks
2.45	Palpation of abdomen reveals a firm, hard spleen, the lower edge of which is at the level of the umbilicus
2.58	X-ray of spleen
3.00	First bleeding. Blood pressure: 120/70
3.03	Platelet count
3.05	11.0 cc. 1.5 per cent vital red solution given intravenously
3.09	Bleeding for blood volume
3.10	Injection of 10.0 cc. liver extract Eli Lilly (or 20 cc. Parke Davis) begun. Blood pressure: 122/68
3.15	Patient complained of severe pain in abdomen, headache, and nausea. No vomiting
3.17	Marked flushing of face has occurred
3.20	Blood pressure: 84/44
3.25	Injection completed. Spleen palpable about 2.0 cm. below the costal margin
3.26	X-ray of spleen
3.28	Bleeding
3.30	Platelet count
3.38	11.0 cc. 1.5 per cent vital red solution given intravenously
3.42	Bleeding for blood volume
3.45	X-ray of spleen
24 hours later x-ray of spleen	

The results obtained in a typical study, using liver extract by intravenous injection as a stimulant to splenic contraction, are shown in Table II. From this table it is evident that without essential change in the total blood volume there was a significant increase in the formed elements of the blood following a shrinkage in the size of the spleen. Twenty-four hours later the spleen had practically regained its original size. Figure 1 presents tracings of the splenic shadow taken from the x-rays and demonstrates the change of size of the spleen. The area of the spleen before injection was 90.3 sq. cm. Immediately after

injection the spleen had contracted to an area of 32.3 sq. cm. Accompanying this contraction there was an increase of 520,000 red blood cells in the erythrocyte count. The hemoglobin increased 15 per cent. At the same time even more marked rises were found in the white count and the platelet count, the increase of the former being 950 cells and of the latter 74,000. Determination of the blood volume after contraction of the spleen showed an increase of the total blood volume of only 200 cc. With this the plasma volume decreased from 65 to 60 per cent. There was also a decrease in the total plasma volume.

TABLE II

Effects of Contraction of the Spleen Induced by Liver Extract

	Area of spleen	Red count	Hemoglobin	White count	Mean corpuscular volume	Color index	Platelet count	Blood pressure
	sq. cm.	*millions*	*per cent*		μ^3			
Patient								
Before injection	90.3	3.72	70	1400	0.870	0.950	49,000	120/70
After injection	32.3	4.24	85	2350	0.865	1.00	123,000	95/60
24 hours later	89.1	3.67	69	1150	0.860	.945	52,000	
Control								
Before injection		2.68	67	4850	1.26	1.25	152,000	115/65
After injection		2.57	66	4500	1.28	1.28	138,000	90/60

	Total blood volume	Total plasma volume	Plasma per cent
	cc.	*cc.*	
Before injection	3,800	2,520	65
After injection	4,000	2,410	60

The fragility of the red cells to sodium chloride solutions was not altered after contraction of the spleen.

A study of the formed elements of the blood following the injection of liver extract intravenously into a control patient with anemia, but without splenomegaly, is also included in Table II. It is evident from these results that there was no appreciable alteration in these elements following this injection. Results similar to those found in Table II have been obtained repeatedly following the intravenous injection of liver extract into the patient with splenomegaly. The degree of contraction of the spleen and the changes in the formed elements of the

blood have varied directly with the degree of contraction obtained in each study. The observations presented here are neither the maximal nor the minimal results obtained, but are taken as the most typical.

The combined results of the alterations of the formed elements in the peripheral blood, obtained after induced splenic contraction in five studies in which liver extract was used as the stimulant, are presented in Table III. The average splenic contraction as shown was from a surface area of 93.6 sq. cm. to an area of 50.0 sq. cm. This is a decrease in the size of the spleen to 53 per cent of its original size. The

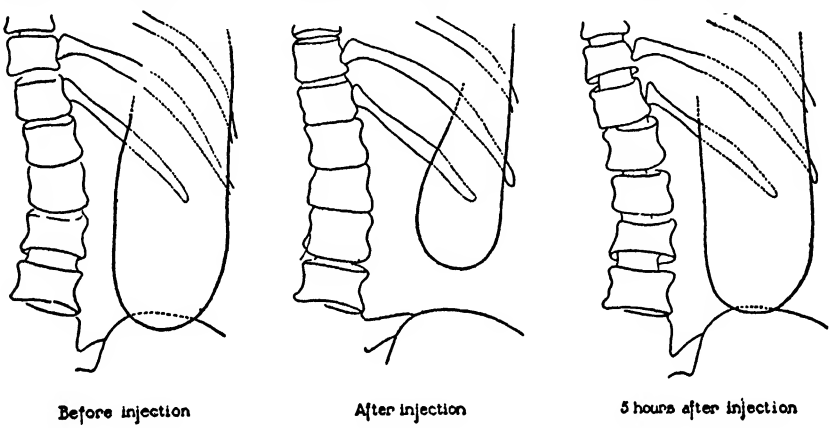

Fig. 1. Shows a series of tracings taken from the x-rays of the spleen demonstrating the size of the spleen immediately before the injection of liver extract, immediately after contraction had occurred, and 5 hours later, at which time the spleen had practically regained its original size.

average increase in the erythrocyte count after contraction was 500,000 red cells, or an increase of 13.9 per cent. Together with this the hemoglobin increase in this series was 10 per cent, or an average increase of 13.7 per cent of the original average hemoglobin value. The white blood cells showed an average increase of 840 cells, or average increase of 51 per cent. The blood platelets showed an average increase of 66,000 or of 105 per cent. It is apparent from this table that the relative increases were far greater for the white cells and the blood platelets than for the erythrocytes; this observation was seen in every study.

These observations suggested the possibility of attempting to evaluate on the human spleen the effects of certain drugs which have been

FIG. 2. This is an x-ray of the spleen taken immediately before the injection of liver extract. In this x-ray the lower border of the spleen is seen beneath the crest of the ilium.

TABLE III

Combined Studies of Splenic Contractions Induced by Liver Extract

	Area of spleen	Red count	Hemo-globin	White count	Platelet count
	sq. cm.	*millions*	*per cent*		
Before injection	93.6	3.62	73	1660	63,000
After contraction	50.0	4.12	83	2500	129,000
Per cent increase		13.9	13.7	51.0	105.0

known to stimulate splenic contractions in animals. Consequently, adrenalin, histamine, and eserine were used to induce splenic contractions. The results obtained following the intramuscular injection of these drugs are presented in Table IV.

FIG. 3. This is an x-ray of the spleen taken immediately after the injection of liver extract, and demonstrates the change in size of the splenic shadow.

As shown in the table a marked contraction of the spleen was noted eight minutes after the intramuscular injection of 1.0 cc. of adrenalin. The spleen contracted from an area of 109.0 sq. cm. to an area of 48.7 sq. cm. One hour later it had increased in size to 82.4 sq. cm. Accom-

panying this contraction there was an increase in the red blood cell count of 650,000 cells, together with an increase of hemoglobin of 19 per cent. In this study the white blood cells showed the maximal increase obtained, namely, 2,600 cells, or an increase of 172 per cent. With this contraction there was also an increase in the platelet count of 68,000.

TABLE IV

Effects of Contraction of the Spleen Induced by Adrenalin, Histamine, and Eserine

	Area of spleen	Red count	Hemoglobin	White count	Mean corpuscular volume	Color index	Platelet count	Blood pressure
	sq. cm	*millions*	*per cent*		μ^3			
Adrenalin								
Before injection	109.1	3.22	63	1500	0.917	0.985	90,000	124/75
8 minutes after injection of 1 cc., I.M.*	48.7	3.87	79	4100	0.925	1.01	158,000	138/80
1 hour after injection ..	84.2							
Histamine								
Before injection	100.8	3.32	65	1650	0.915	0.985	68,000	122/78
7 minutes after injection of 1 mgm.. I.M.	75.5	3.67	70	2700	0.915	0.960	124,000	106/52
1 hour after injection .	93.5							
Eserine								
Before injection .	97.5	3.37	64	1250	0.900	0.955	96,000	128/70
40 minutes after injection of 2.4 mgm., I.M	73.6	3.36	64	1100	0.890	0.955	88,000	145/75

* I.M. –intramuscularly.

Splenic contraction was also induced by the intramuscular injection of 1.0 mgm. of histamine. Seven minutes after this injection the spleen had contracted from 100.8 sq. cm. to 75.5 sq. cm. In one hour it had increased in size to 93.5 sq. cm., that is, almost to its original size. With this contraction there was an increase in the red cell count of 350,000 red cells, with a 5 per cent increase of hemoglobin. The white blood cells increased by 1,050 cells and the platelet count was increased by 56,000 platelets. As in the previous studies the white blood cells and platelets showed a greater proportional increase than the red cells and the hemoglobin.

FIG. 4. This x-ray of the spleen was taken 5 hours after the contraction had occurred. It demonstrates the return of the spleen to its original size.

TABLE V

Differential White Counts

	Contraction induced by liver extract		Contraction induced by adrenalin	
	Before	After	Before	After
	per cent	*per cent*	*per cent*	*per cent*
Polymorphonuclears	46	72	44	57
Eosinophils				1
Small lymphocytes	51	22	52	37
Large lymphocytes	2	2	2	2
Monocytes	1	4	2	3

The intramuscular injection of 2.4 mgm. eserine sulphate also induced a splenic contraction. This contraction did not occur until 40 minutes after the injection. The surface area of the spleen decreased from 97.5 sq. cm. to 73.6 sq. cm. As seen in Table IV there was no appreciable alteration in the formed elements of the blood accompanying this contraction. This was the only study in which no significant alteration of the formed elements of the blood was obtained with an induced splenic contraction. When the parasympathetic nervous system was blocked by giving atropine intramuscularly no contraction followed the injection of eserine.

Differential blood counts are found in Table V. There was a relative and absolute increase in the polymorphonuclear cell count after the induced splenic contraction. Accompanying this there was a relative decrease in the lymphocyte count. These variations were found with each splenic contraction studied. No abnormal cells were seen in the smears taken either before or after the contraction. The reticulocyte count showed no variation.

The blood pressure was followed in each study, as may be seen in Tables II and IV. A fall in blood pressure was invariably obtained following the intravenous injection of liver extract. A similar fall of blood pressure occurred after the injection of histamine. In the studies in which adrenalin and eserine were used as the stimulant to contraction, there was invariably a rise in blood pressure.

DISCUSSION

Before discussing the data presented in this paper it may be well to point out that while certain changes in blood cytology took place as a result of the induced splenic contractions, the results may not be indicative of the function of the normal spleen, since it may be recalled that the patient showed both an anemia and a splenomegaly. Obviously such an abnormal organ could store a much greater quantity of blood or cells than could a spleen of normal size. Furthermore, the degree or ratio of contraction obtained could scarcely be expected to occur with the normal spleen. Thus it might be thought that the pathological conditions might negate or partially invalidate the significance of these observations as regards the normal physiological function of the organ as a blood reservoir. This matter cannot be definitely settled at this

time, but the authors prefer the interpretation that the present circumstances constitute merely an exaggeration of the normal.

The mechanism of the contractions is not clearly understood. Dale and his co-workers (8) isolated histamine from alcoholic extracts of fresh liver. Histamine present in the liver extract given intravenously might then be the substance responsible for the induction of the splenic contraction. Similar effects, namely a splenic constriction, were produced with liver extract and with a drug stimulating the sympathetic nervous system, adrenalin. In these two types of experiments, there was a lowering of the blood pressure with the liver extract and a rise in blood pressure accompanying the contraction induced by adrenalin. This would seem to eliminate the fall in blood pressure as a significant occurrence. Furthermore, whereas there occurred a peripheral vasodilation in the study with liver extract, and a peripheral vasoconstriction in the contraction induced with adrenalin, it may be safely stated that peripheral vasodilation had little or no effect on the production of the alteration in the blood elements.

The contraction induced by histamine may have been due to the direct action of the drug on the unstriated musculature of the organ, or it may have been the response of the spleen to shock produced by the drug.

A drug which stimulates the parasympathetic nervous system, namely, eserine, also gave a splenic contraction. It may be pointed out that the contraction was smaller than that obtained by other means, and that there was no alteration in the blood elements accompanying the change in size of the spleen.

Blood volume determinations showed very little alteration. There may have been at other times a greater increase in blood volume not detected by the method used. Vital red cannot be used repeatedly, intravenously, in clinical studies.

Perhaps the most striking fact among these results is the disproportionate increase in cell counts. Thus in each of the studies with liver extract and also with the other stimulating drugs the increase in the number of white blood cells was over threefold that of the red blood corpuscles, while the proportionate increase of the blood platelets was almost invariably greater than that of the white blood cells. No matter what mechanism was involved in the addition of these cells

to the peripheral blood, these ratios may represent the relative proportions of these cells available in the splenic reservoir. This being the case the spleen may be considered as a potential and readily available source, not only of red blood corpuscles, but of white blood cells and blood platelets as well. It is not inconceivable that the spleen functions as a reservoir for these cellular elements.

SUMMARY

1. In a patient who showed both anemia and a splenomegaly it was possible to induce [and observe] a marked contraction of the spleen by the intravenous injection of liver extract, and the intramuscular injection of adrenalin, histamine, and eserine. This contraction was observed.

2. Accompanying these induced splenic contractions there occurred marked increases in the number of cellular elements of the circulating blood. The relative proportion of the various cellular components suggests that the blood of the splenic reservoir has a cytological composition in which the relative proportion of cells is different from that of the circulating blood.

3. These experiments make it obvious that the spleen must be considered not only as a reservoir of red blood cells, but that it can also store proportionally greater numbers of white blood cells and bloob platelets.

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THE PATHOLOGY OF THE BONE MARROW IN SPRUE ANEMIA*

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INTRODUCTION

The study of bone marrow by means of specimens removed during life has been applied frequently to forms of anemia other than those associated with tropical sprue. Ghedini,[1] Morris, and Falconer,[2] and Zadek[3,4] all studied the bone marrow of living patients with pernicious anemia. It remained for Peabody[5,6] to apply more modern knowledge to the study of the finer structure of bone marrow. In his final paper he showed most clearly that in pernicious anemia the marrow was hyperplastic during relapse and tended to return to normal during remission. Although the existence of a relation between the macrocytic anemia of sprue and of pernicious anemia has become manifest, existing observations of the bone marrow in sprue with anemia are few in number and confusing. Ashford[7] briefly reported findings in specimens of tibial marrow studied during life by another worker. Both hyperplastic and aplastic histological pictures were found. No photographs are shown nor are any descriptions of the histological changes given. The author's conclusions as to the marrow pathology seem to be colored largely by existing hypotheses of marrow function based on the response of the peripheral blood to therapy. Mackie and Fairley[8] described the changes found mainly in the femoral and tibial marrows of a group of autopsied cases of

* These observations were carried out in 1931 by the Commission of the Rockefeller Foundation for the Study of Anemia in Puerto Rico.

sprue. They noted in certain instances hyperplastic marrow, such as is found in pernicious anemia. In others fatty marrow was found, with rare, circumscribed areas of activity. Still other marrows showed a type of gelatinous degeneration. These various manifestations were considered to represent various stages, ranging from hyperplastic "active" marrow to atrophic "exhausted" marrow. The fact that the marrow of the long bones is constantly fatty in character under normal conditions and is the last to respond with hyperplasia was not emphasized. Their briefly reported histological studies added little to the descriptions of the gross picture as regards the character of the cellular reaction. However, the red femoral marrow of one patient was stated to have exhibited "production of numerous megaloblasts as well as nucleated red cells."

The most careful and detailed studies of the bone marrow in sprue are those reported by Krjukoff.[9] This worker studied biopsy material from the ribs of sixteen patients. He was impressed by the constancy with which he found a megaloblastic change of the marrow with the production of "lymphoid erythroblasts" of all sizes, megaloblasts, and normoblasts. He does not mention any cases in which an inactive marrow was found. The uniformity of the pathological alterations found is in striking contrast to the observations of other investigators and conforms much better to what is now known to be the effect of adequate specific therapy.

The divergent results of these studies of sprue have, at least in part, been due to the fact that specimens from comparable areas of the marrow were not studied. Piney[10] has shown that only the ribs, flat bones, and vertebral column normally contain active cellular marrow in adult life. Peabody[5] has emphasized the fact that the marrow of a long bone, such as the tibia, is not necessarily homogeneous in structure and that the pathological process there does not necessarily correspond in extent and degree to that in the marrow of other bones. On the other hand, his own studies of pernicious anemia were carried out on specimens of tibial marrow because he felt that it was simpler in structure and hence presented alterations easier to interpret than the more complicated marrow of flat bones or sternum. After the clarification of the underlying cellular changes of the marrow in pernicious anemia as a result of Peabody's work, it appeared logical

to us to examine a site of normally active marrow in sprue. It was felt that in this way early alterations might be observed more readily and conclusions drawn with more certainty as to the presence of hyperplasia or aplasia of the functional elements. For these reasons this communication is based largely upon examination of specimens of sternal bone marrow removed at operation.

Methods

Twenty-two patients with sprue anemia of a macrocytic type were studied. In certain instances specimens were removed both before and after remissions, in others during the height of the reticulocyte response to liver therapy. The eventual death of three patients made it possible to compare the postmortem pathological picture with that seen during life. In still another group abnormalities of the clinical course were correlated with the histopathological alterations of the marrow.

The procedure of removing the specimens of sternal marrow was carried out in the operating room under complete surgical precautions for asepsis. A longitudinal midline incision about 4 cm. long was made from the third to the fifth costal cartilages, and was extended down to the periosteum. Two incisions were made in the periosteum, each about 1 cm. long and at right angles to each other. The periosteum was carefully retracted to expose an area of bone about 1 cm. in diameter. With a small crown trephine the outer table of bone was removed as a button about 0.4 cm. in diameter. With a bone curette enough marrow was removed to ensure the inclusion in microscopic sections of actual marrow tissue. The bone button was replaced and hemorrhage from the bone stopped with bone wax. The periosteum was brought together with No. 0 plain catgut. One or two subcutaneous stitches of the same suture material were used to approximate the subcutaneous tissue and the skin was closed with interrupted silk sutures. A dry dressing applied with considerable pressure from heavy adhesive straps was kept in place for twenty-four hours.

The tissue was fixed immediately in Zenker's fluid. After the usual steps of dehydration the tissue was embedded in paraffin and cut at 6 microns without decalcification. Mallory's phosphotungstic acid hematoxylin, eosin-methylene blue and the Giemsa stain were employed. Certain blocks were cut serially.

RESULTS

The results of the microscopic examination of the bone marrow are summarized in the table and will be discussed below. In addition a few illustrative cases are briefly cited, and the microscopic picture described in detail.

Cellular Composition of the Sternal Bone Marrow in Untreated Cases of Sprue

CASE 1. *Clinical History:* The patient was a Puerto Rican housewife, 47 years of age. There was a history of fairly typical sprue for one year, with loss of weight, gastro-intestinal disturbance, glossitis, and anemia. Before the biopsy the patient had been treated for sixteen days with autolyzed yeast, to which she showed a slight reticulocyte response and slight clinical improvement. By the time of the biopsy the lingual and gastro-intestinal symptoms were somewhat improved without significant changes in blood values.

On the day of the biopsy, Aug. 26, 1931, examination of the blood showed: Red blood cells 2,150,000 per cmm.; hemoglobin 54 per cent (Sahli); color index 1.25; mean corpuscular volume 120 cu. μ; leukocytes 8100 per cmm.; reticulocytes 1.8 per cent; icterus index 2. The blood smear was consistent in appearance with the macrocytic anemia of sprue.

Bone Marrow Biopsy: The tissue appeared only moderately cellular and contained quite a large number of bone spicules. It was red-brown in color. The microscopic appearance is illustrated in Figure 1. A considerable amount of fat is present. The number of cells is distinctly greater than that observed in normal sternal marrow. Groups of from six to eight megaloblasts are seen with round nuclei containing rather heavy masses of chromatin and occasionally in mitosis. Certain cells of this general type are larger, with a considerable amount of rather pale, basophilic, cytoplasm. The latter is the type of cell that is present in such large numbers in the bone marrow in pernicious anemia in relapse. Smaller cells, diffusely scattered, with round nuclei containing dense masses of chromatin are numerous. These have a heavily basophilic cytoplasm that varies greatly in amount, though it is never so great as in the megaloblast. These cells are considered to be erythroblasts of varying degrees of maturity. A considerable number of normoblasts is present, many more than are seen in the marrow of pernicious anemia in relapse or in marrows from patients with sprue of a more severe degree. Megakaryocytes are

present in about normal numbers, as are cells of the granulocytic series. Many sinusoids are closed.

Comment: Subsequent heavy dosage with liver extract administered intramuscularly failed to effect a reticulocyte rise or improvement in blood values. When ferric ammonium citrate was administered, however, a distinct reticulocyte rise appeared and subsequently a return to normal blood levels resulted. In this marrow the frequent occurrence of normoblasts suggests a deficiency of iron as well as of liver extract, which is confirmed by the fact that both liver extract and iron were required to bring about improvement. In contrast to the findings in the bone marrow of Case 2, the histological changes are slight. It is conceivable that this is the earliest change, since the illness of the patient was short, and the blood values were at a relatively high level.

CASE 2. *Clinical History:* The patient was a male journalist, 60 years of age, who had suffered from gastro-intestinal disturbance, glossitis, and weakness for ten years. For the ten days preceding the biopsy the patient was treated with 6 gm. of ferric ammonium citrate daily without effect.

On the day of the biopsy, Sept. 8, 1931, the blood examination showed: Red blood cells 1,760,000 per cmm.; hemoglobin 49 per cent (Sahli); color index 1.39; mean corpuscular volume 126 cu. μ; white cells 6200; reticulocytes 1.4 per cent; icterus index 5. The blood smear was typical of the macrocytic anemia of sprue.

Bone Marrow Biopsy: The marrow did not appear remarkable at operation. Some increase in cellular over bony tissue was present and the color was distinctly reddish. The microscopic appearance is illustrated in Figure 2. A moderate amount of fat is present, up to thirty cells per high power field. Between the fat cells are masses of cellular tissue. The vast majority of the cells present are megaloblasts, some arranged in groups and others scattered diffusely throughout. They are larger, in general, than are those seen in Case 1 and have considerably more cytoplasm. Mitoses are frequent. Many erythroblasts of varying degrees of maturity are seen. The relative number of normoblasts is strikingly decreased. A few cells of the granulocytic series are present. The marrow is similar to that seen in pernicious anemia in relapse, except for the lack of complete replacement of fat by cellular tissue. The sinusoids are open and contain many adult red cells. The uniformity of the cellular picture is most striking. Megakaryocytes are sharply reduced in number.

TABLE I

Table Showing Blood Picture and Bone Marrow Histology in Macrocytic Anemia of Sprue

Blood picture								Bone marrow						Comment
Case No.	Red blood cells in millions	Hemoglobin *per cent*	White blood cells in thousands	Reticulocytes *per cent*	Mean corpuscular volume in cu. μ	Icterus index units	Color index	Biopsy or postmortem	Bone source	General cellularity	Megalo-blasts and erythro-blasts	Normo-blasts	Fat	
Untreated Cases														
1	2.15	54	8.1	1.8	130	2	1.25	Biopsy	Sternum	++	++	++	++	Very early though definite megaloblastic preponderance (Fig. 1).
2	1.76	49	6.2	1.4	126	5	1.39	"	"	+++	+++	+	+	Moderate megaloblastic proliferation and preponderance (Fig. 2).
3	1.41	40	3.3	3.2	126	4	1.42	"	"	++++	++++	−	−	Intense megaloblastic proliferation and preponderance (Fig. 3).
4	1.42	48	2.2	6.6	140	40	1.69	"	"	++++	++++	−	−	" " " " "
5	0.98	28	2.2	0.8	143	25	1.43	"	"	++++	++++	−	−	" " " " "
6	2.51	55	3.3	4.8	101	2	1.10	"	"	++++	++++	−	−	" " " " "
7	1.96	46	9.8	1.4	114	2	1.17	"	"	++++	++++	−	−	" " " " "
8	1.92	42	4.8	2.0	102	5	1.10	"	"	++++	++++	−	−	" " " " "
9	2.67	66	7.4	2.2	119	3	1.24	"	"	++++	++++	−	−	" " " " "
10	1.15	34	5.1	3.8	131	30	1.48	"	"	++++	++++	−	−	" " " " "
11	2.52	63	8.1	3.2	128	3	1.25	"	"	++++	++++	−	−	" " " " "
12	3.12	73	7.5	1.6	107	2	1.14	"	"	++++	++++	−	−	" " " " "
13	1.25	29	1.8	1.0	104	18	1.16	"	"	++++	++++	−	−	" " " " "
14	1.68	55	5.0	4.6	134	5	1.63	"	"	++++	++++	−	−	" " " " "
15	2.40	54	1.9	0.4	113	40	1.12	"	"	++++	++++	−	−	" " " " "
16	1.46	40	3.0	0.1	121	5	1.37	"	"	+++	++	+	+	Moderate megaloblastic proliferation and preponderance.

17	1.05	29	2.9	8.8	157	12	1.38	Postmortem	Sternum and femur	++++	++++	–	–	Phagocytosis of red cells present (Fig. 6).
18	2.86	68	7.6	1.6	119	3	1.19	"	"	++++	++++	–	–	Sternal marrow red, femoral partly red. Lung abscess. Microscopically very loose structure.
19	0.97	19	7.3	4.5	103	30	0.98	"	"	++++	++++	–	–	Brick red marrow sternum and upper third of femur. Transfusion. Microscopic structure confused. Phagocyted erythrocytes.

Treated Cases

20	0.94	19	3.6	4.4	115	10	1.00	1st biopsy	Sternum	++++	++++	+	–	Before remission. Many megaloblasts, few normoblasts (Fig. 4), almost no fat cells.
20	2.97	60	3.3	4.2	100	2	1.01	2nd biopsy	"	++	+	+++	++	20 days after intramuscular liver extract. Predominant normoblasts (Fig. 5).
21	0.83	26	3.0	14.0	162	3	1.57	Biopsy	"	++	++	+	+	9th day oral liver extract. During reticulocyte rise. Megaloblasts separating into clumps. Normoblasts appearing.
22	1.40	29	2.3	21.6	114	6	1.04	1st biopsy	"	+++	++	++	+	9 days after liver extract intramuscularly. During reticulocyte rise. Decrease in megaloblasts. Increased normoblasts.
22	2.69	55	2.8	2.0	99	3	1.02	2nd biopsy	"	+	+	++++	+++	30 days after liver extract. Late remission. Marrow picture approaches normal, except for increased normoblasts.

Comment: The administration of ferric ammonium citrate effected no improvement. Liver extract given orally brought about a slight reticulocyte rise and by subsequent parenteral administration restoration of normal blood values was slowly attained. This result could perhaps have been prognosticated from the changes present in the marrow. That the response was a slow one might be associated with the fact that although the cell type was uniform, complete replacement of fat had not taken place. This marrow is intermediate in type between that of Case 1, where megaloblastic activity was mild and early, and much fat present, and that of Case 3, in which a picture quite similar in all respects to that of pernicious anemia was seen.

CASE 3. *Clinical History:* The patient was a Puerto Rican housewife, 60 years of age, who had suffered from malnutrition for one year, and during the past three months from glossitis, diarrhea, and edema of both feet. There had been considerable loss of weight.

On the day of the biopsy, Aug. 26, 1931, the blood examination was as follows: Red blood cells 1,410,000 per cmm.; hemoglobin 40 per cent (Sahli); color index 1.4; mean corpuscular volume 125 cu. μ; white blood cells 3300 per cmm.; reticulocytes 3.2 per cent; icterus index 4. The blood smear was typical of the macrocytic anemia of sprue.

Bone Marrow Biopsy: Grossly the marrow appeared dark red and hyperplastic. Definite replacement of bone spicules had taken place. Little fat was seen. As shown in Figure 3, microscopic examination bears out this impression. Almost no fat is present, averaging fewer than one fat cell per high power field. The tissue is rather vascular and extremely cellular. Masses of large megaloblasts are present. These cells are somewhat irregular in size and outline. The nuclei are rounded or oval and contain rather little chromatin, that present being clumped in irregular masses. The cytoplasm is slightly basophilic and irregular in outline. Smaller clumps of cells of somewhat different character are seen. These cells have round nuclei with heavy masses of chromatin. The cytoplasm varies in amount and is deeply basophilic. These cells are considered to be erythroblasts. Very few normoblasts are seen. A moderate number of megakaryocytes is present, as is some myelopoiesis with all stages in the development of the granulocytic cell series.

Comment: The picture is one of diffuse megaloblastic hyperplasia,

similar in nature to that seen in pernicious anemia in relapse. As might be expected with the type of bone marrow change described, a prompt reticulocyte response followed the intramuscular injection of liver extract. This response was followed by eventual restoration of normal blood values.

In the three cases just described different degrees of the same fundamental histological change have been observed. The presence of such definite variations in the intensity of the process, even in the sternal marrow, is considered to be a partial explanation of the failure of many investigators to find active marrow in the long bones of certain autopsied cases of sprue anemia. Presumably, in such instances, the pathological change has failed to extend to the long bones, though strikingly present in the sites of most active blood formation where the earliest changes would be expected to appear. In no case examined, and many, as may be seen from the table, had anemia of an extreme degree, was the sternal bone marrow atrophic. This is in agreement with the observations of Krjukoff and quite opposed to the statements of those who have confined their studies to the marrow of the long bones.

The Effect of Therapy on the Cellular Composition of the Sternal Bone Marrow in Sprue

Hitherto the marrow in cases of sprue anemia has not been studied before and after treatment capable of producing reticulocyte rises and restoration of normal blood values. Two cases of the present series were subjected to sternal punctures at different stages of the disease and the histological pictures compared. The changes were similar in both cases.

CASE 20. *Clinical History:* The patient was a male Puerto Rican, 35 years of age, who entered the hospital complaining of weakness of six months' duration. For four months he had suffered from watery diarrhea and glossitis. The day before the first biopsy he received a transfusion of 200 cc. of whole blood and an injection of an effective extract derived from 100 gm. of liver.

On the day of the first biopsy, Aug. 6, 1931, a blood examination showed: Red blood cells 940,000 per cmm.; hemoglobin 19 per cent (Sahli); color index 1.0; mean corpuscular volume 115 cu. μ; white blood cells 3600 per cmm.; reticulocytes 4.4 per cent; icterus index 10.

First Bone Marrow Biopsy: In the gross the tissue was soft, deep red, and had definitely replaced a large part of the bony trabeculae. Histologically, as shown in Figure 4, all resemblance to normal marrow structure is lost. No fat is present. The tissue is an almost solid mass of large, pale cells, with nuclei containing scattered masses of chromatin. Their outline is irregular, the cytoplasm moderately basophilic, and the cells are in close apposition. These cells are considered to be megaloblasts. Scattered diffusely throughout in large numbers are somewhat smaller cells with round nuclei containing dense and heavy chromatin. The cytoplasm of these cells is small in amount and is somewhat more deeply basophilic. Many mitoses are present. Nucleated red cells are almost entirely absent. Myelopoiesis is slight. Only a limited number of megakaryocytes are present. The vascular channels are obscured by the enormous cellular overgrowth. The whole picture is quite consistent with that seen in severe pernicious anemia.

The effect of liver extract on this patient was entirely similar to that occurring in Addisonian pernicious anemia. In response to the single injection of liver extract derived from 100 gm. of liver the reticulocytes reached a peak of 38 per cent on the fifth day and the blood values rose rapidly, as similar therapy at ten day intervals was maintained.

Twenty days after the first biopsy and after remission had occurred, though complete restoration of the blood levels to normal had not taken place, a second biopsy was performed. On the day of the second biopsy, Aug. 26, 1931, the blood examination showed: Red blood cells 2,970,000 per cmm.; hemoglobin 60 per cent (Sahli); color index 1.02; mean corpuscular volume 100 cu. μ; white blood cells 3300 per cmm.; reticulocytes 4.2 per cent; icterus index 2.

Second Bone Marrow Biopsy: Histologically, the picture is quite different from that of the first biopsy, as shown by a comparison of Figure 4 with Figure 5. Fat cells are present up to ten to twelve per high power field. A few large pale megaloblasts with irregular nuclei are present in groups of three or four. The solid background of these cells seen in the first specimen is completely replaced by sheets of normoblasts that greatly outnumber any other cell type present. Vascular channels are open, myelopoiesis is proceeding, and a fair number of giant cells may be seen. Clearly, the administration of

effective therapy caused a maturation of megaloblasts to normoblasts with the production of a bone marrow morphology more nearly approaching the normal and so resembling the transition observed during the treatment of pernicious anemia.

Phagocytosis of Erythrocytes by Bone Marrow Cells in Sprue

The presence of erythrocytes in the cytoplasm of large cells of the bone marrow in pernicious anemia has been described frequently in specimens of marrow removed at autopsy. At a time when current views associated pernicious anemia with increased blood destruction, based upon the observations of the increased serum bilirubin content and an output of bile pigment greater than normal in the excreta, Peabody considered that phagocytosis might be of significance in the production of the anemia. In an early paper Peabody and Broun[11] gave a detailed description of the phagocytic picture in pernicious anemia, as seen in tissue fixed postmortem, and compared it with postmortem material from other types of disease. Although phagocytosis occurred, especially in infectious diseases, it was apparently greater in cases of pernicious anemia where death occurred in an acute stage. Later observations of biopsy material, however, failed to reveal erythrocytes in the cytoplasm of large marrow cells and led to the conclusion that the cytological appearances, which were supposed to indicate the phagocytosis of red blood cells during life, were really postmortem, or at least terminal phenomena.

Study of postmortem specimens of bone marrow from two cases of sprue anemia in Puerto Rico revealed the same phenomenon, so frequently observed in marrows of patients dying of pernicious anemia. In both instances erythrocytes are seen in the cytoplasm of large bone marrow cells, as shown in Figure 6. In both of these specimens the architecture of the marrow is seriously distorted, as compared with tissue removed at biopsy. In the latter, closely packed masses of cells, uniform in size and shape and easily identifiable, with well marked vascular channels and stroma structure are seen. In the postmortem specimens the structure is extremely loose and almost unrecognizable. There is an enormous variation in size and shape of the cells. Some are very large and have an irregular, slightly basophilic cytoplasm, which contains large numbers of erythrocytes. Since this was not

observed in any of the specimens removed at biopsy the conclusion is unavoidable that it is a postmortem change. In neither case was the body kept more than eight hours before the tissue was fixed, although lack of facilities for refrigeration and the heat of the climate may have accelerated a postmortem alteration. Peabody states that the cells that ingest erythrocytes in the bone marrow of pernicious anemia are clasmatocytes. He quotes the observation of Rich[12] that clasmatocytes may ingest red cells when grown *in vitro*. From the observations here presented no conclusion as to the nature of the phagocytic cell can be drawn. Only further evidence, based on supravital and tissue culture study of material removed at autopsy, can be expected to solve the problem.

DISCUSSION

The material presented indicates that, as concerns the fundamental histopathological picture of the bone marrow during exacerbation, during remission, and at postmortem, the changes accompanying the macrocytic anemia of tropical sprue are similar to those found in pernicious anemia, as described by others.

Peabody confirmed the previous observation of Zadek that during remission the bone marrow became less cellular; and for the first time accurately described the microscopic appearances both during remission and in relapse. He considered the hyperplasia occurring in relapse to be an extensive proliferation of primitive marrow cells, chiefly megaloblasts, associated with a relative decrease in the other elements, including the fat cells. Remission was shown to be characterized by a picture more nearly normal, with few megaloblasts, a relative increase of normoblasts and mature red blood cells, and the reappearance of fat. The anemia of relapse was explained by the functional ineffectiveness of the marrow resulting from the failure of the megaloblasts to form mature erythrocytes. In the sternal marrow of nineteen patients with untreated sprue the same fundamental pathological change was found, an increase in number and size of the megaloblasts, a decrease in the amount of fat present, and in the number of megakaryocytes and cells of the granulocyte series. The number of normoblasts was strikingly small in comparison to the number of megaloblasts. The differences in the bone marrow changes

reported by previous observers can thus apparently be explained by the fact that only the marrow of the long bones was studied. Beyond question the extent of marrow involvement is greater in pernicious anemia than in certain cases of sprue, but the changes occurring in the normally active marrow of the adult, obtained here by sternal biopsy, present a uniform picture.

Furthermore, the effect of specific therapy in sprue anemia is followed by the same maturation of megaloblasts to normoblasts and restoration of normal morphology of the marrow, as occurs in pernicious anemia. This fact parallels the similarity of the blood changes in the two conditions. Although, as a rule, the effect of liver extract on the lingual and gastro-intestinal symptoms is as striking as in pernicious anemia, the hematopoietic response is frequently not so marked for a given blood level in sprue as in pernicious anemia. This is probably to be explained, at least partly, on the basis of the fact that in pernicious anemia the megaloblastic hyperplasia involves a greater amount of the normally inactive bone marrow. It is also clear that in sprue a combination of therapy with iron, as well as with liver extract, is necessary in many cases, a fact that correlates with the findings in the bone marrow of certain patients, as illustrated by Case 1. Despite this fact, the basic similarity of the blood pictures and of the changes in the active portions of the bone marrow in relapse and in response to similar therapy would seem to be strong evidence for a similar etiological mechanism. Observations have been made by Castle and Rhoads[13] that indicate the macrocytic anemia of sprue is mainly the result of a deficiency similar to that existing in pernicious anemia, although brought about in a somewhat different manner.

CONCLUSIONS

1. Observations on the bone marrow of sprue anemia made on tissue obtained at biopsy in different stages of the disease show that the changes are similar to those of pernicious anemia.

2. During relapse the essential change is a proliferation of megaloblasts and suppression of maturation to the normoblast stage.

3. During remission the marrow tends to return to normal with a great increase in the number of normoblasts and mature red cells in the marrow.

4. Phagocyted erythrocytes were observed in the bone marrows removed at autopsy but not in those removed during life.

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DESCRIPTION OF PLATES

Plate 130

Fig. 1. Case 1. Sternal marrow removed at biopsy from patient with macrocytic anemia of sprue of moderate degree and duration before remission produced subsequently only after addition of iron to liver extract therapy. Note presence of much fat, numerous normoblasts and moderate numbers of megaloblasts. Giemsa stain. × 1500.

Fig. 2. Case 2. Sternal marrow removed at biopsy from patient with macrocytic anemia of sprue. Note presence of fat and moderate megaloblastic preponderance. Cells of the myeloid series are present in considerable numbers. Giemsa stain. × 1000.

Plate 131

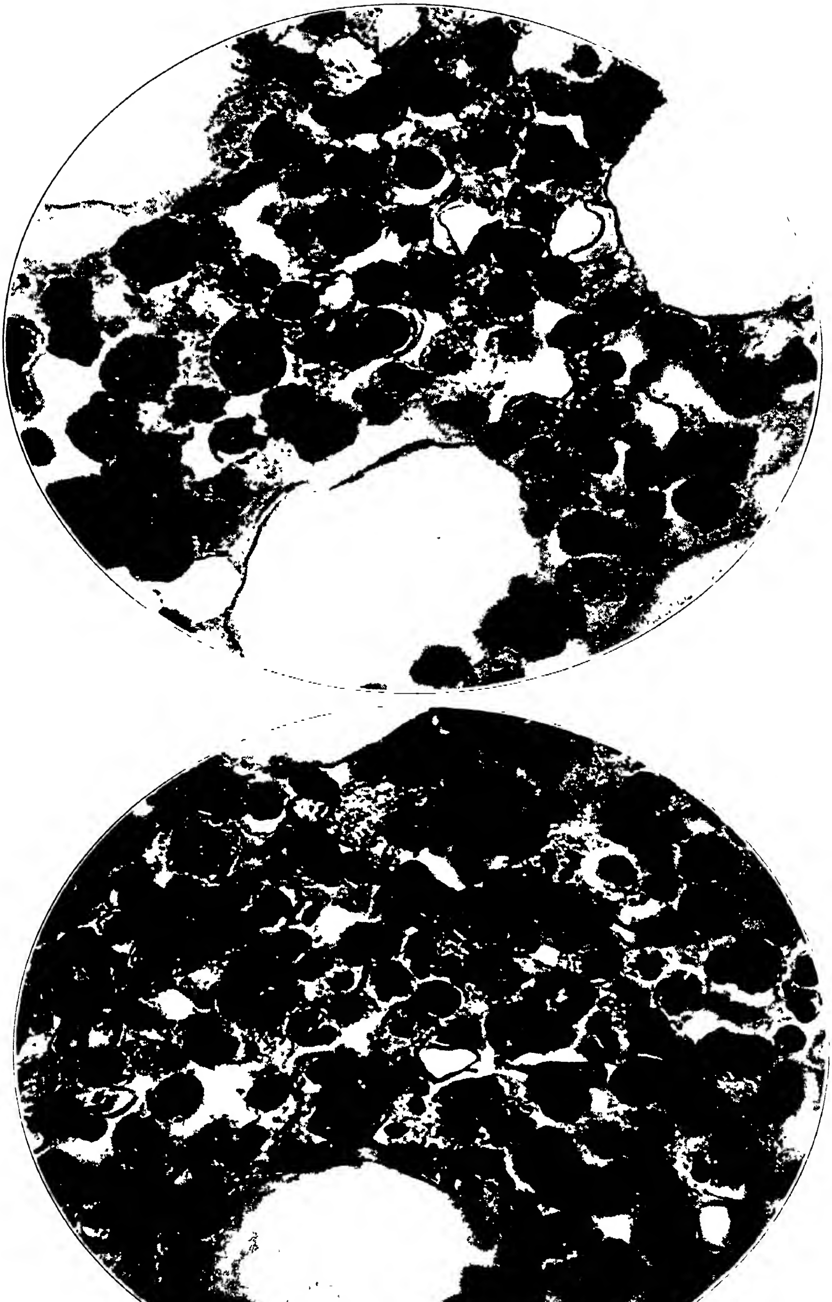
Fig. 3. Case 3. Sternal marrow removed at biopsy from patient with severe macrocytic anemia of sprue. Note almost total absence of fat and intense megaloblastic proliferation with relatively few normoblasts and cells of myeloid series. Giemsa stain. × 1500.

Fig. 4. Case 20. Sternal marrow removed at biopsy from patient with severe macrocytic anemia of sprue before typical remission produced with liver extract. Note absence of fat with increased cellularity due largely to intense megaloblastic proliferation with occasional mitotic figures. Giemsa stain. × 1000.

Plate 132

Fig. 5. Case 20. Second sternal marrow removed at biopsy twenty days after injection of active liver extract, which produced a typical reticulocyte crisis. Note the decreased number of megaloblasts and the normoblastic preponderance. Giemsa stain. × 1000.

Fig. 6. Case 17. Sternal marrow removed at autopsy from patient dying of sprue with severe macrocytic anemia. Note loss of structural details and presence of phagocyted erythrocytes. Giemsa stain. × 1000.



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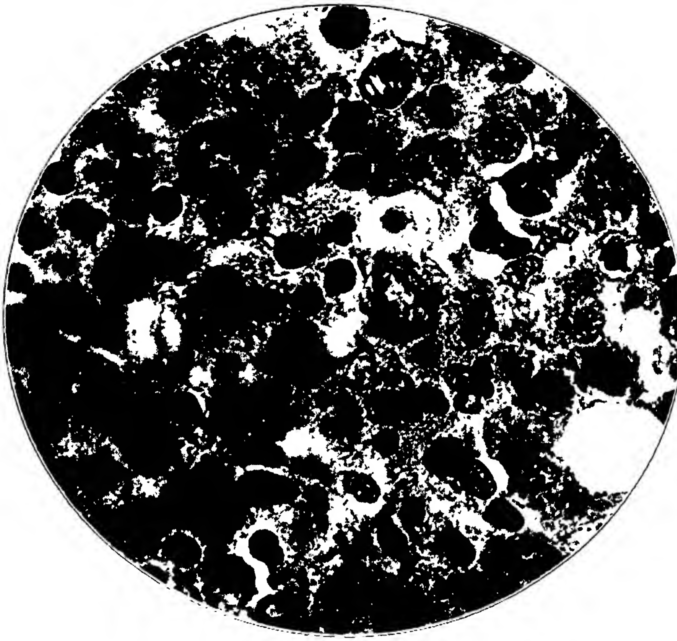

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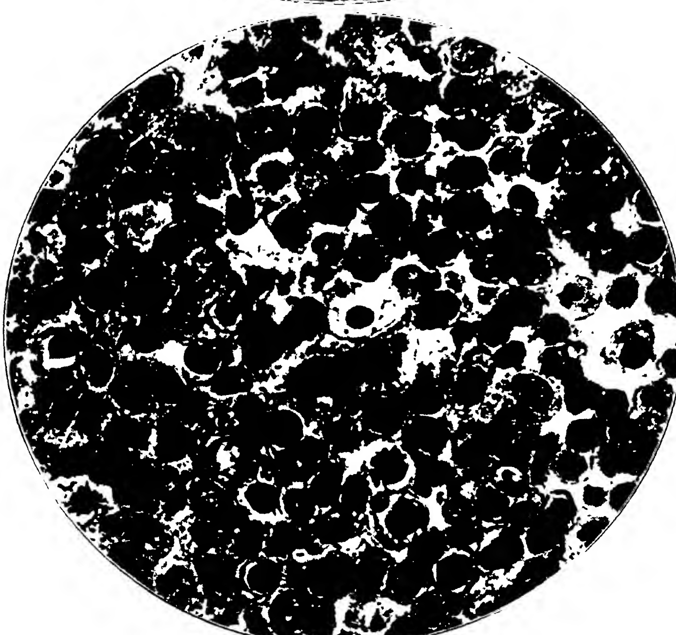

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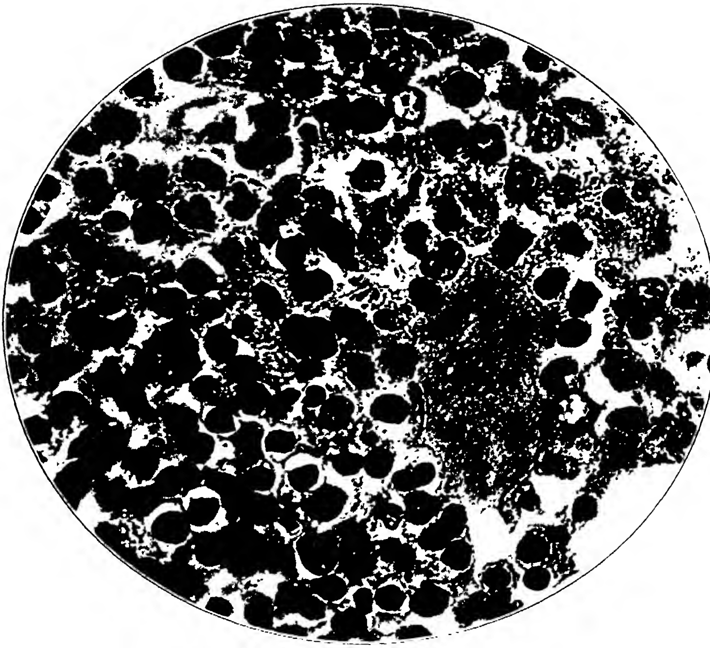
Rhoads and Castle

Bone Marrow in Sprue Anemia



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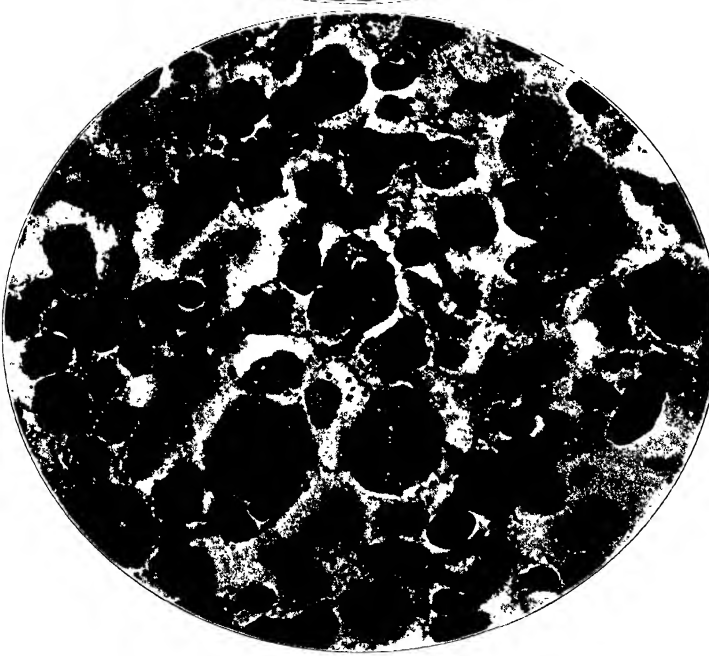

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Rhoads and Castle

Bone Marrow in Sprue Anemia

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INFECTIOUS PAPILLOMATOSIS OF RABBITS

BY RICHARD E. SHOPE, M.D.

WITH A NOTE ON THE HISTOPATHOLOGY

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PLATES 33 TO 35

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Our attention was recently called to a disease occurring in wild cottontail rabbits in northwestern Iowa.[1] Rabbits shot there by hunters were said to have numerous horn-like protuberances on the skin over various parts of their bodies. The animals were referred to popularly as "horned" or "warty" rabbits.

Warts from a naturally occurring case of the disease in Iowa were obtained and sent to the laboratory in sterile 50 per cent glycerol. These glycerolated warts furnished us our original material for investigation. A little later, in a shipment of a dozen wild cottontail rabbits from southern Kansas, three were found to be affected with the same wart-like disease. To date, out of 75 wild cottontail rabbits received from Kansas eleven have been found to carry one or more warts. These eleven animals serve as the basis for our description of the naturally occurring disease.

Description of the Naturally Occurring Disease

In wild cottontail rabbits the presence of warts has caused no apparent discomfort in our experience and induced no demonstrable evidence of generalized illness. Most of the animals were sacrificed, shortly after their arrival, for pathological material, but four, kept under observation for 7 weeks or longer, at no time appeared ill and were in good physical condition when finally killed. The number

[1] We are grateful to Mr. T. A. McKichan of Cherokee, Iowa, who first told us of the disease, and to Mr. Clifford Peck of Cherokee and Mr. Earl Johnson of Rago, Kansas, who furnished us with naturally occurring cases of the disease.

of warts on individual animals in our series varied from one to ten in all cases except one. The exceptional animal was almost literally covered with warts and these, when removed at autopsy, were sufficient to fill a 200 cc. flask. The most common sites for the warts were on the inner aspect of the thighs, the abdomen, or about the neck and shoulders. Individual warts varied somewhat in size but the greater number were from 0.5 to 1 cm. in diameter and from 1 to 1.5 cm. in height. They were black or grayish black in color, well keratinized, and the upper surfaces were frequently irregular or fissured. They were roughly oval in shape when viewed from the side (Fig. 1). The bases, in most instances, were narrower than the mid-portion. The lateral surfaces of the warts appeared vertically striated because each individual wart was composed of closely packed and almost homogeneous vertical strands of tissue. On cut section, an average wart had a white or pinkish white fleshy center, and the upper portion and its lateral surfaces were grayish black and keratinized. The vertically striated structure was particularly evident on cut section. Attachment to the skin was loose as evidenced by the ease with which warts were knocked or pulled off when animals were handled. Warts removed in this way left a rather freely bleeding surface which in most instances healed without complication; sometimes a second wart appeared at the same site.

Experimental Transmission

No difficulty has been encountered in transmitting the condition to either domestic or wild cottontail rabbits when materials from naturally occurring cases have been employed. The method used is, in brief, as follows:

Either freshly removed warts or those that have been stored in 50 per cent glycerol at refrigerator temperature are ground to a fine paste with sterile sand and physiological saline in a mortar. More physiological saline is added to make a 3 to 5 per cent final suspension. Such a suspension is then centrifuged and the supernatant fluid, which is only slightly turbid, is removed and used for inoculation. Suspensions prepared in this way remain infectious for at least a month when kept at refrigerator temperature.

Inoculation by scarification was regularly performed in these experiments. Rabbits to be inoculated were shaved on the abdomen or sides and lightly scarified either by needle or by rubbing the shaven skin with a moderately coarse grade of sterilized sandpaper. To obtain discrete warts the former method was employed, while scarification with sandpaper was used when a confluent and massive growth of warts was desired. The scratches were made only deep enough to cause a barely perceptible oozing of blood-tinged fluid. A small amount of the infectious suspension was immediately applied by dropping it from a syringe, and this fluid was rubbed well into the scarifications by means of a spatula or the flat handle of a scalpel. The area thus inoculated was allowed to become almost dry before the animal was released and put into its cage.

Course of the Experimental Infection

The disease produced by experimental infection of either wild cottontail or domestic rabbits followed a typical course when infectious material from naturally occurring cases was employed. The period of time elapsing between inoculation and the first appearance of macroscopically detectable lesions varied from 6 to 12 days with an average of slightly more than 8 days. This variation in the incubation period was probably due more to differences in the potency of the infectious suspensions than to differences in the resistance of individual animals, for when the same suspension was employed in inoculating a series of rabbits the incubation period was the same in the entire series.

The first detectable lesions consisted in minute, barely visible elevations along the lines of scarification. These, on the 1st day, appeared macroscopically to be tiny vesicles, an appearance not supported by histological examination. By the 4th or 5th day the lesions were more definite and numerous and were usually pink in color. They were approximately 1 mm. in diameter and height and had lost their delicate vesicular appearance. From this stage on, growth was constant although its rapidity varied considerably. From the 16th to the 20th day after their first appearance, the lesions were approximately 3 mm. in diameter and height if they were isolated on the shaven areas or, if confluent, they constituted a more or less solid mass of rough wrinkled pinkish keratinized tissue 3 to 4 mm. in thickness. They had, by this time, acquired a definitely warty appearance, their surfaces were keratinizing, and their sides exhibited the peculiar type of perpendicular striations seen in the naturally occurring warts. The warts, whether separate or confluent, continued to increase in size for an indefinite period, and as the lesions became older they became more and more cornified until finally the upper portions were very hard. The lower portions, however, usually remained fleshy to the touch. At 6 weeks, individual warts or confluent masses were from 1.2 to 1.5 cm. in height; the skin on the portion of the body upon which they were developing had become pendulous and was thrown into large stiff folds. Animals sacrificed at this time exhibited an enormously increased blood supply in the subcutaneous tissue underlying the warts. In spite of the great size of many of our experimentally produced wart masses, the animals showed no loss in weight and the entire course of the disease was free from any general clinical evidence of illness. In their gross appearance the experimental warts in both domestic and wild rabbits have been identical with those seen in the naturally occurring disease. Photographs of experimentally produced warts are given in Figs. 2 to 4.

Experimental warts, as well as those occurring naturally, appear to remain stationary when they reach 1 to 1.5 cm. in height. One of our rabbits, however, at present, 6 months after inoculation, is carrying a large wart mass which in places is 3 cm. in height. With two exceptions, we have seen no warts retrogress in animals infected in the usual way. In the exceptional animals, one a wild and the other a domestic rabbit, warts developed slowly after an unusually long incubation period. They reached a maximum height of only 2 to 3 mm. between 30 and 40

days after inoculation and in 60 days had completely disappeared. Both of these animals were inoculated with the same infectious suspension and were the only ones so inoculated. In no animal in which growth of warts took place in the usual fashion and in which the lesions reached a thickness of 1 cm. or more have we seen any evidence of retrogression. To date we have had experimentally infected animals under observation for 6 months only. While there has been no evidence of retrogression of the papillomata except in the cases mentioned, there has also, so far, been no evidence that the lesions of prolonged standing are acquiring malignant properties. Animals are being held under observation to determine what the ultimate fate of the papillomata will be.

HISTOPATHOLOGY

By E. WESTON HURST

Histology of Naturally Occurring Warts

The growths consist of a number of closely adjacent, branching filiform processes of epidermis with very narrow connective tissue cores (Fig. 5). This formation suggests simultaneous growth from many centers, with resulting lateral pressure; at the margins the growth bulges over the neighboring normal skin. Transition from the normal epithelium at the edge is relatively abrupt through a narrow zone showing rapid thickening of the epithelial layers.

In the area of new growth the germinal cells of the Malpighian layer may be taller and narrower than normal, giving a palisade effect; mitoses are always present and often numerous, and are found in layers four or five cells removed from the corium. Melanin pigment is much more abundant than in the surrounding skin. The polygonal cell layer is greatly increased in depth, as is the granular layer; the cells vary greatly in size and include a variable number of monster cells with enormous vesicular nuclei. There is no definite eleidin layer; the granular layer passes more or less abruptly into the horny layer, which is often imperfectly keratinized and shows skeleton cell outlines with pyknotic nuclear remains (Fig. 6).

In many cases the tips of the longer papillae are in a necrotic condition, probably determined by the obviously deficient blood supply; that necrosis occurs suddenly is shown by the detection of remains of mitotic figures in the dead tissue. Acute inflammation may supervene locally. Evidence of mild inflammation in the skin beneath the wart is furnished by the presence, in small numbers, of lymphocytes, plasma cells, and polymorphonuclear leucocytes in the subcutaneous tissues.

No definite cellular inclusions have been recognized. Spherical or ovoid eosinophilic structures exist in the outer layers of the wart; at first sight intracytoplasmic, they can usually on careful examination be seen to lie between the cells or in indentations of the cell membrane. They can be traced into the deeper

layers almost to the germinal layer, and appear to originate from necrosis of individual cells at this level.

Histology of Experimental Warts

As early as the 7th day after inoculation, in a series showing macroscopic lesions on the 10th day, slight thickenings of the epithelium may be detected microscopically in the inoculated area. By the 9th day, quite definite localized thickenings composed largely of rapidly multiplying cells, differing but little from normal epithelial cells, are present. Though to some extent elevated above the general skin surface, the cellular masses project more into the corium beneath, where by lateral extension they come to underlie the normal epithelium (Fig. 7). The granular layer is several times the normal in depth, but as yet there is no excess keratinization. The growth is obviously primarily epithelial.

Sections of warts macroscopically visible for 5 days reveal an almost continuous sheet of thickened epidermis extending over the inoculated area. Mitoses are abundant even some distance from the germinal layer in cells containing many keratohyaline granules. Keratinization of the newly formed cells is beginning and at this stage is more perfectly accomplished than later.

Gradually the warts project as papillae from the surface (Fig. 8). The polygonal cells show greater variation in size, keratinization is less perfect, pigmentation occurs in excess, and all the features of the spontaneous growth are faithfully reproduced (Fig. 9). Necrosis of the tips of the papillae may occur as early as the 20th day when the warts are thickly set on the inoculated area. Growth continues actively for at least 91 days, the most advanced case of the histological series, at which period mitoses are still numerous. No signs of spontaneous regression have been noticed. There is no observable difference in warts induced in wild and in domestic rabbits.

In the wild rabbit, removal of the main mass of the wart may be followed by regeneration of an identical structure. Warts induced in a wild animal already infected show no histological variation from the spontaneous growths.

Throughout the experimental series, a variable degree of inflammatory infiltration with mononuclears and polymorphonuclear leucocytes is evident in the corium; this is never intense.

Viability of the Wart-Inducing Agent in Glycerol

Warts from naturally occurring cases stored in equal parts of glycerol and 0.9 per cent NaCl solution at refrigerator temperature for as long as 106 days remain fully infectious. Warts stored for longer periods have not been tested for infectivity.

Filtrability of the Wart-Inducing Agent

Warts to be used as a source of infection in the filtration experiments were removed from the 50 per cent glycerol in which they had been stored and were washed in three changes of sterile physiological saline. They were then minced with sterile scissors, ground in a mortar with sterile sand, and suspended in sufficient physiological saline to make an approximately 5 per cent suspension. Suspensions thus prepared were cleared by centrifugation. The decanted supernatant fluid was usually almost water-clear with only a faint opalescence, and for this reason was rapidly filtrable. 1 cc. of a broth culture of *B. prodigiosus* was added to each 15 to 20 cc. of fluid just before it was passed through Seitz or Berkefeld filters. The resulting filtrates were tested for sterility in 1.5 cc. amounts. All filtrates recorded were bacteriologically sterile.

The results of the filtration experiments are summarized in Table I. Warts produced by filtrates, recorded in Table I as positive, were as extensive and characteristic as those in the control animals which had been inoculated with unfiltered suspensions. Furthermore, when domestic rabbits were used as the test animals, filtration, especially through Berkefeld V or N candles, instead of prolonging the incubation period as might be expected because of some possible removal of the filtrable agent by absorption on the filter surface, usually had either no effect or shortened the period. In wild rabbits, from the limited data at hand, it would seem that filtration resulted in a slight prolongation of the incubation period. From the data recorded in Table I it can be concluded that the etiological agent causing warts in rabbits readily passes Berkefeld filters, of V, N, or W porosity but does not regularly pass a Seitz filter when two pads are employed. Filtration through a Seitz filter, using one pad, allowed not only the virus to pass but also *B. prodigiosus.*

No extensive attempts to cultivate visible microbial forms from filtrates of proven infectivity were made. However, during the investigation active filtrates have been cultured repeatedly in plain and blood broth and on plain and blood agar and such cultures have remained sterile both as regards the test organism, *B. prodigiosus*, or any other visible bacterial form. While no special media have been employed in these attempts to demonstrate the bacteriological sterility of active filtrates, the results obtained using the media mentioned above, considered with the fact that sections of actively growing warts or films of

TABLE I

Filtration Experiments

Experiment No.	Filter	Time of filtration	Amount of filtrate	Maximum negative pressure	Rabbit No., inoculated	Result: wart formation on inoculated skin	Incubation period
		min.	*cc.*	*cm. Hg*			*days*
1	Berkefeld V	2	30	62	DR* 621	Positive	6
	Unfiltered suspension				DR 620	"	6
2	Berkefeld V	0.75	30	62	DR 644	"	6
	" N	1	19.5	62	DR 575	"	6
	" "	1	19.5	62	WR† 634	"	9
	" W	2	10.5	62	DR 613	"	8
	" "	2	10.5	62	WR 632	"	9
	Unfiltered suspension				DR 666	"	9
	" "				WR 637	"	7
3	Berkefeld V	0.50	29	62	DR 640	"	7
	Unfiltered suspension				DR 681	"	10
4	Seitz (2 pads)	2	30	Positive pressure	DR 711	Negative	
	" (2 ")	2	30	" "	DR 725	"	
	Berkefeld V	2.5	30	62	DR 729	Positive	12
	" "	2.5	30	62	WR 733	"	12
5	" N	0.5	34	62	DR 790	"	9
	" W	3	27	62	DR 789	"	9
	Unfiltered suspension				DR 791	"	9
6	Seitz (2 pads)	2	34	Positive pressure	DR 793	"	15
	Berkefeld N	1.5	29	62	DR 788	"	7
	" "	1.5	29	62	DR 794	"	7
	" W	3	29	62	DR 784	"	11
	" "	3	29	62	DR 795	"	11
	Unfiltered suspension				DR 792	"	9

* DR = domestic rabbit.

† WR = wild rabbit.

active unfiltered infectious suspensions have failed to reveal the presence of any constant perceptible microbial form, would seem clearly to indicate that no visible organized agent is etiologically essential to the wart production.

Heat Resistance of the Wart-Inducing Agent

The method just described for preparing wart suspensions for filtration was employed in the heating experiments to be outlined. Only the slightly turbid supernatant fluid of centrifuged physiological saline suspensions of glycerolated

TABLE II

Heat Resistance of the Wart-Inducing Agent

Heated for 30 min.	Rabbit No., inoculated	Result: wart formation on inoculated skin	Incubation period
°C.			*days*
45	716, right side	Positive	10
55	715	"	11
60	746, left side	"	7
60	757 " "	"	7
60	713	"	10
60	772	"	8
60	726	"	9
60	785	"	8
65	716, left side	"	10
65	748 " "	"	9
65	759 " "	"	8
65	752 " "	"	8
65	778 " "	"	8
65	738	"	8
65	781, left side	Negative	
67	753 " "	Positive	26 (only 5 warts)
67	779 " "	"	20 (developed well for) 10 days, then retrogressed)
67	782, left side	"	20 (developed poorly and retrogressed)
70	753, right side	Negative	
70	778 " "	"	
70	781 " "	"	
73	779 " "	"	
73	782 " "	"	
73	783	"	
75	746, right side	"	
75	757 " "	"	
75	752 " "	"	
75	777	"	
75	780	"	
85	748, right side	"	
85	759 " "	"	

warts was used. The fluid to be heated was sealed in sterile glass ampoules and completely submerged in the water bath for the 30 minute period during which it was exposed to a given temperature.

The results of the heating experiments are given in Table II.

The data recorded in Table II indicate that the activity of the wart-inducing agent is unaffected by temperatures of 65°C. or below for ½ hour but is completely destroyed by temperatures of 70°C. or higher. Heating to 67°C. for 30 minutes, while not completely inactivating the wart-producing agent, did exert a deleterious influence on it. This was evidenced by a marked prolongation of the incubation period in rabbits infected with material heated to this temperature and by scant takes and early retrogression of the resulting warts. It was of interest to note in this respect that the heating of suspensions at temperatures from 45–65°C., instead of lengthening the incubation period in inoculated animals, often shortened it as compared with that shown in animals receiving the unheated control suspensions.

In some of the heating experiments opposite sides of a single rabbit were used to test two suspensions, with adequate care that material from one side did not contaminate the other side. In earlier carefully controlled experiments in which both sides of an animal were shaved and scarified but only one side inoculated with an infectious suspension, it was shown that warts developed only on the inoculated side. In the experiments in Table II, in most instances, inoculations were arranged in such a way that only one side of the animal developed warts.

The thermometer used in these experiments was compared with a standard thermometer and was found to give readings 0.2°C. below those of the standard instrument. This small correction has not been made in the data recorded in Table II.

Routes of Infection

Only the method of inoculation by scarification has yielded constant results in our hands. Inoculation intravenously with infectious Berkefeld filtrates, after first abrading an area of the skin of the abdomen with a sterile needle, led to infection of the abraded areas in two out of four cases. Of the two positive animals, one, a wild rabbit, developed only a single wart; while the other, a domestic rabbit, de-

veloped four warts on the abraded area and two on the back of the neck. The incubation period in both of these cases was over three times as long as that of the control animals infected by scarification. At autopsy, all four intravenously inoculated animals were free from visceral pathology ascribable to the wart-inducing agent. Inoculations of either wild or domestic rabbits intraperitoneally, subcutaneously, intratesticularly, or intracerebrally, with filtrates of proven infectivity on scarification, have yielded entirely negative clinical and pathological results. About 50 per cent of the intradermal inoculations resulted in wart formation although in these instances the warts appeared not at the point where the inoculum had been deposited but at the point where the needle had pierced the epidermis and where some of the inoculum had leaked from the needle tract. The incubation period of warts produced in this way was always longer than when infection had been accomplished by scarification.

Resistance of Infected Rabbits to Reinfection

In a series of 123 wild and domestic rabbits inoculated with suspensions of known infectivity, we have encountered no animal that was naturally immune.

One of the two rabbits in which warts underwent complete retrogression was tested and found to be resistant to reinfection. The serum of this animal, however, when mixed in equal parts with an infectious suspension, failed to neutralize the wart-inducing agent. It did prolong the incubation period considerably.

Ten domestic rabbits carrying warts of various ages have been tested for immunity to reinfection. Five resisted reinfection successfully, while the remaining five, after unusually long incubation periods, finally developed warts at the sites of their new inoculations. These warts were much less numerous and slower in growth than those in the control animals. The time elapsing between the primary infection and the attempt at reinfection was apparently of little importance, for two animals were found to be susceptible to reinfection 76 days after their primary inoculation while one animal was completely resistant to reinfection 31 days after its primary inoculation. Two rabbits tested 14 days after their primary inoculation and 6 days after the first appearance of warts possessed some resistance which was evidenced by the fact that no warts appeared at the sites of their new inoculations for

24 days, whereas the incubation period in the control animals was 8 days.

Three wild rabbits that were carrying warts when received from Kansas were tested for immunity. All three were still susceptible, although the incubation periods were markedly lengthened. One experimentally infected wild rabbit has been found to be immune to reinfection.

Neutralizing Properties of the Sera of Infected Rabbits

Sera from wild rabbits, either naturally or experimentally infected, as well as the sera from experimentally infected domestic rabbits, have been found to contain antibodies effective against the wart-inducing agent. Most of such sera have neutralized it completely, so that warts failed to develop in animals inoculated with mixtures of serum and the infectious agent; in the others, partial neutralization was evidenced by a doubling or trebling of the incubation period.

For these tests the usual virus neutralization technique was employed. Equal parts of serum and infectious suspension were mixed and stored overnight (17 hours) in the refrigerator. The control consisted of equal parts of infectious suspension and normal rabbit serum. Rabbits were inoculated with these mixtures on the freshly scarified skin in the usual way, using one shaven side for the control inoculation and the other for the neutralization test, performing both inoculations on the same rabbit and thus avoiding possible individual variations in resistance.

From the above experiments it is evident that an active wart infection in rabbits not only renders them completely or partially resistant to reinfection but also that it evokes antibodies, demonstrable in their sera, capable of completely or partially neutralizing the wart-inducing agent.

Wart-Producing Agent Not Immunologically Related to the Viruses Causing Infectious Fibroma or Myxoma

In earlier experiments (1), a benign fibroma-like new growth of rabbits caused by a filtrable virus was found capable of establishing a resistance in rabbits to fatal infection with the otherwise uniformly deadly virus of infectious myxoma. To explore the possibility of an immunological relationship between the wart-producing agent and the benign fibroma virus or the fatal myxoma virus, a number of experiments were conducted. It was found that rabbits infected with the

wart-producing agent and carrying large warts at the time of testing were fully susceptible to both the fibroma and the myxoma viruses. No alteration of the normal course of either of these diseases was observed as the result of previous infection with the wart-producing agent. Conversely, rabbits recovered from infection with fibroma virus and demonstrably immune to reinoculation with that virus were still fully susceptible to infection with the wart-producing agent. Rabbits immunized against infectious myxoma by preliminary infection with fibroma virus and subsequent inoculation with *Virus myxomatosum*, and possessing demonstrable virucidal antibodies for *Virus myxomatosum*, were also still fully susceptible to infection with the wart-producing agent. These experiments indicate that the wart-producing agent is not immunologically related to either of these viruses.

It may be noted here that in the original glycerolated wart material obtained from Iowa both the wart-producing agent and the virus of infectious fibroma were present. The latter was easily separated from the former by testicular passage through domestic rabbits. The strain of fibroma virus thus isolated was typical in all major respects of the original strain described earlier (2), and like it was capable of protecting rabbits against fatal infection with *Virus myxomatosum*.

Attempts to Transmit the Wart-Producing Agent in Series through Rabbits

In all, twenty-six domestic and wild rabbits have been inoculated in the usual way with suspensions prepared from experimentally engendered domestic rabbit warts ranging in age from 1 to 116 days. Not only did all such inoculations yield negative results but the animals, when subsequently tested, were found to be still fully susceptible to infection with the wart-producing agent from wild rabbit papillomata. On the other hand, either naturally occurring or experimentally produced warts from wild rabbits proved readily transmissible to either wild or domestic rabbits. Warts from nine naturally occurring cases of the disease in wild rabbits have been tested and all found to be infectious for both wild and domestic rabbits. In like manner, experimentally produced warts from nine wild rabbits have been tested for infectivity. Eight of these proved infectious for either domestic or wild rabbits while the warts from one proved to be non-transmissible.

We have not yet attempted to pass the wart-producing agent through a long series of wild rabbits but in the course of obtaining fresh infectious material it has at present reached its third serial passage. In spite of the fact that the agent cannot be propagated in series through domestic rabbits, it is probable that it can be passed indefinitely in series through wild rabbits and that any of these serial wild rabbit passages can be used in infecting domestic rabbits.

No attempt has so far been made to transmit the domestic rabbit warts by means of tissue grafts, although in a small number of experiments freshly prepared cell-containing suspensions of young actively growing papillomata from domestic rabbits have yielded negative results when inoculated intracutaneously or subcutaneously into domestic rabbits. Instead, it has seemed best to study the rabbit papillomata first as an infectious process caused by a filtrable agent and to determine, if possible, why this agent should be readily transmissible in series when inducing warts in wild rabbits and non-transmissible when inducing similar growths in domestic rabbits.

That the degree of maturity of the warts in domestic rabbits at the time that attempts were made to transmit them in series was not a determining factor is indicated by the fact that warts taken at intervals of 6 to 8 days, from their first appearance until they were 116 days old, yielded no successful infections.

Domestic rabbit warts glycerolated for varying periods of time were repeatedly tested for infectivity to determine whether or not glycerol storage has an activating effect on the agent as it does on herpes virus of low activity (3–5). The results of these experiments were all negative.

In a series of experiments conducted before the presence of neutralizing antibodies in the blood serum of wart-bearing animals had been demonstrated, it was found that when an inactive domestic rabbit wart suspension was mixed with an equal part of a suspension prepared from wild rabbit warts of known infectivity, the resulting mixture was either completely non-infectious or the incubation period was prolonged and the resulting warts few in number as compared with control animals. This suggested the presence in warts from domestic rabbits of an inhibitory substance similar to that found by Sittenfield, Johnson, and Jobling (6) and Murphy, Helmer, Claude, and Sturm (7) in fowl tu-

mors. In the light of subsequent experiments in which the sera of wart-bearing rabbits were found to neutralize partially or completely the wart-producing agent, it seems possible that the inhibitory properties observed in non-infectious domestic rabbit wart suspensions might in reality have been due to contained humoral antibodies. A point of argument against this belief is that, while humoral antibodies were demonstrable in the sera from both infected wild and domestic rabbits, only the domestic rabbit warts possessed demonstrable inhibitory properties. We have as yet made no systematic attempt to render experimental domestic rabbit warts infectious by removal of a hypothetical inhibitory substance. We have tried, however, to infect rabbits with inactive experimental domestic rabbit wart suspensions that had been heated to 60°C. for 30 minutes in the hope that that temperature might inactivate the possible inhibitor without affecting the wart-producing agent, with suspensions prepared from domestic rabbit wart cells that had been washed repeatedly and sufficiently to remove all freely soluble humoral antibody, and with Berkefeld filtrates of inactive wart suspensions. All three of these procedures yielded completely negative results. Both the Iowa and the Kansas strain of the disease were used in these attempts to transmit warts in series through domestic rabbits.

DISCUSSION

The absence of significant visible bacterial forms in highly active wart-producing suspensions together with the ready filtrability of the etiological agent and the inability to cultivate, on lifeless media, any visible microbial form from demonstrably active filtrates; the agent's ability to transmit in series through wild rabbits; its glycerol resistance; its ability to induce in its hosts an immunity which is constant although of variable degree; and its apparent tropism for one type of tissue place this agent in the filtrable virus group.

The non-transmissibility of the agent in series through one of its demonstrably susceptible hosts, the domestic rabbit, is not a characteristic of most of the known virus diseases. An analogy, however, is to be found in the group of filtrable fowl tumors. Des Ligneris (8), working with Rous Sarcoma 1 of chickens, has found that while both turkeys and guinea fowls are susceptible, transmission through these two alien species is limited to two successive serial passages. Similarly,

Andrewes (9) has found that while Rous Sarcoma 1 will produce fatal metastasizing tumors in its first pheasant passage it cannot be transmitted in its characteristic form from pheasant to pheasant.[2] It seems probable that the domestic rabbit (genus *Oryctolagus*) is sufficiently distantly related to the wild cottontail rabbit (genus *Sylvilagus*) to behave towards infection with a filtrable new growth of wild rabbit origin in much the same manner as do turkeys, guinea fowls, and pheasants towards infection with a filtrable chicken tumor.

Another property of the wart-producing agent that is unusual among viruses causing diseases in animals is its resistance to heat. Suspended in 0.9 per cent NaCl solution it proved capable of withstanding a temperature of 65°C. for 30 minutes in sealed ampoules without apparent damage to its wart-producing properties. Virus heated to 67°C. for 30 minutes, while still active, produced, in our limited number of experiments, warts which either developed scantily or retrogressed after a few days' growth. We are aware of no other animal virus which will withstand so high a temperature in the moist state; most are completely inactivated at much lower temperatures. However, among the plant viruses, which are on the whole as susceptible as animal viruses to the effects of heat, there are several which withstand heating to 65°C. or more (10). The virus of tobacco mosaic is an example of a typical plant virus that is relatively heat resistant (11). For this reason it does not seem necessary to consider seriously the possibility that the unusual heat resistance of the wart-producing agent eliminates it from classification as a virus.

The not infrequent shortening of the incubation period in animals inoculated either with virus heated to from 45–65°C. or with virus that had been filtered through Berkefeld V or N candles cannot be explained. Removal of an inhibiting agent by these two procedures is suggested by the data.

In the gross and histologically, the warts of rabbits described in this paper are typical of virus-produced papillomata (12–15) as known in man, cattle, and dogs. It has not been previously observed in studies of mammalian warts of this kind that an epithelial neoplastic process of identical gross and histological appearance can be induced in two ani-

[2] Andrewes has recently reported the successful serial passage of Rous Sarcoma 1 through pheasants (Andrewes, C. H., *J. Path. and Bact.*, 1933, **37,** 17).

mal species, in one of which the condition is not only transmissible in series, but transmissible by cell-free filtrates, and in the other of which it is not transmissible at all. Here then in what is certainly a single clinical entity are examples of the two extremes of neoplastic processes considered from the standpoint of transmissibility. In the wild rabbit the papillomata can be initiated by inoculating the animal with a filtrable agent and they are transmissible in series by inoculation with filtered or unfiltered virus. From an etiological standpoint, then, the wild rabbit warts are analogous to the chicken tumors which by some are not considered as true representatives of neoplastic processes simply because they are transmissible by cell-free filtrates. Thus the wild rabbit papilloma represents the one extreme of a tumor induced by an infectious agent which can be separated from the cells and some of whose properties can be studied.

The other extreme is exemplified by the papillomata induced in domestic rabbits which, while initiated by the same virus, have so far resisted transmission either to domestic or wild rabbits. These are thus analogous to many of the tumors of mammals which cannot be transmitted in series by the usual methods of transplantation. No objection to the eligibility of the domestic rabbit warts for consideration as neoplastic processes could be raised on the grounds that a causative agent distinct from the proliferating cells can be discriminated. A study of this epithelial new growth in domestic rabbits without knowledge of its causation would probably lead an investigator to classify it as one of that large group of so called "spontaneous" mammalian tumors that are non-transmissible. It would not even be suspected that the papillomata had been caused by a filtrable virus of wild rabbit origin.

The question which is naturally brought to mind by the experiments of des Ligneris (8) and Andrewes (9) with fowl tumors and our own with rabbit warts is whether certain "spontaneous" non-transmissible or not readily transmissible tumors may not originally have been caused by viruses which produce transmissible tumors in some other species. A careful study, from this point of view, of the causes underlying the non-transmissibility of these various tumors may bring to light new knowledge of the etiology of neoplastic processes in general, especially in the group of mammalian tumors which are either entirely

non-transmissible or transmissible only by viable cell-containing grafts.

SUMMARY

A papilloma has been observed in wild cottontail rabbits and has been found to be transmissible to both wild and domestic rabbits. The clinical and pathological pictures of the condition have been described. It has been found that the causative agent is readily filtrable through Berkefeld but not regularly through Seitz filters, that it stores well in glycerol, that it is still active after heating to 67°C. for 30 minutes, but not after heating to 70°C., and that it exhibits a marked tropism for cutaneous epithelium. The activities and properties of the papilloma-producing agent warrant its classification as a filtrable virus.

Rabbits carrying experimentally produced papillomata are partially or completely immune to reinfection and, furthermore, their sera partially or completely neutralize the causative virus. The disease is transmissible in series through wild rabbits and virus of wild rabbit origin is readily transmissible to domestic rabbits, producing in this species papillomata identical in appearance with those found in wild rabbits. However, the condition is not transmissible in series through domestic rabbits. The possible significance of this observation has been discussed. The virus of infectious papillomatosis is not related immunologically to either the virus of infectious fibroma or to that of infectious myxoma of rabbits.

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EXPLANATION OF PLATES

Plate 33

Fig. 1. Spontaneous wart on the thigh of a wild rabbit. The vertically striated appearance of the lateral surfaces can be seen. The upper portion of the growth is hard and well keratinized.

Fig. 2. Experimental warts on the abdomen of a domestic rabbit showing individual discrete wart formation in an animal infected following scarification by needle. These warts are 23 days old.

Fig. 3. Same animal as shown in Fig. 2. The warts are now 52 days old and, considered individually, are accurate reproductions of the spontaneous warts seen in wild rabbits.

Fig. 4. Experimental warts on the abdomen of a domestic rabbit showing massive confluent wart formation in an animal infected following scarification with sandpaper. These warts are 118 days old and are firmly keratinized.

Plate 34

Fig. 5. Section of a spontaneous wart in a wild rabbit. The long, branching papillae are capped by an enormous amount of keratinized material. Iron alum hematoxylin and eosin. × 10.4.

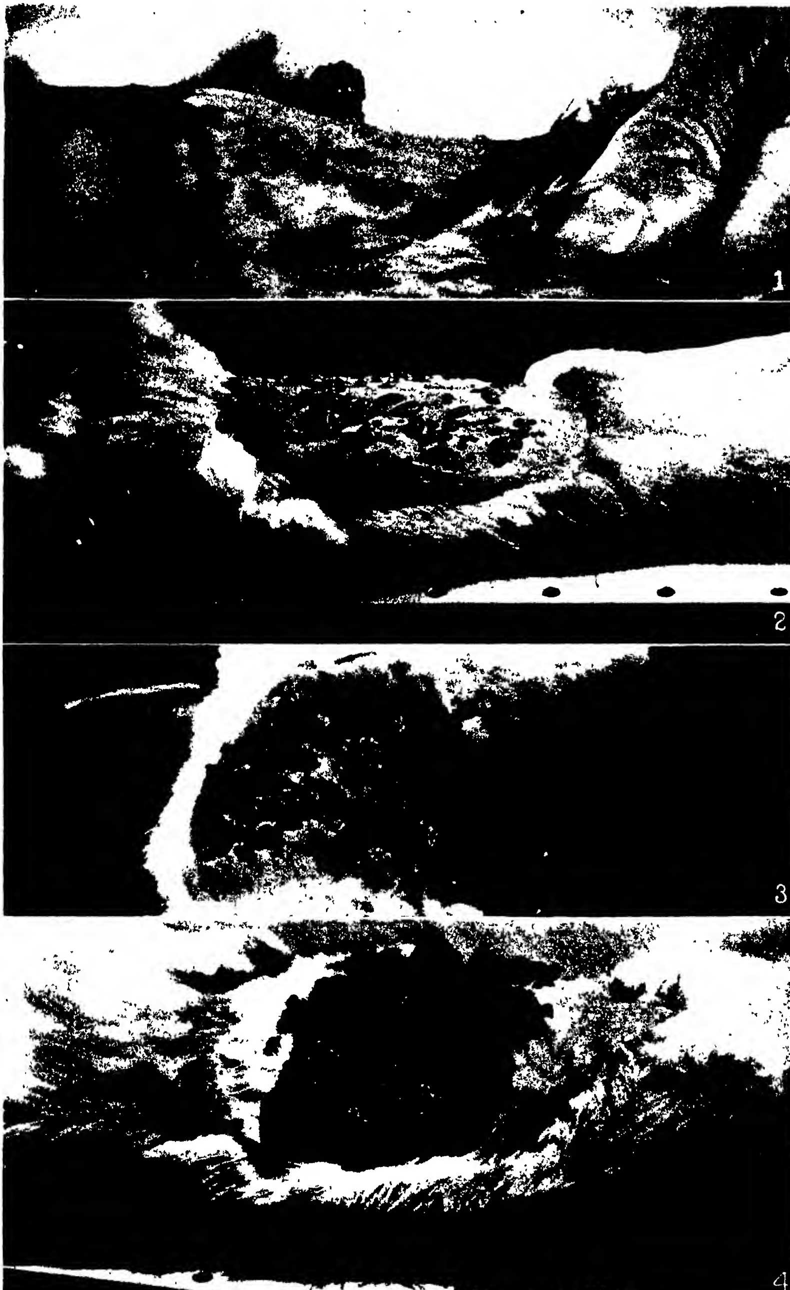
Fig. 6. Higher power of a spontaneous wart showing the tall and narrow germinal cells, the great thickness of the polygonal cell and granular layers, and the imperfectly keratinized surface layer. Iron alum hematoxylin and eosin. × 168.

Plate 35

Fig. 7. Section of an experimental wart in a domestic rabbit 3 days after its appearance. The mass of proliferating epithelium lies partly below the level of the normal epithelium, which it underlies at the margins. As yet there is no excess keratinization. Iron alum hematoxylin and eosin. × 33.

Fig. 8. Section of an experimental wart in a domestic rabbit 18 days after its appearance. The epithelium is greatly thickened and the new growth projects considerably from the surface. Iron alum hematoxylin and eosin. × 77.

Fig. 9. Still later stage of the experimental disease in a domestic rabbit (36 days). The features of the spontaneous growth are by now fairly faithfully reproduced. Iron alum hematoxylin and eosin. × 26.



(Shope: Infectious papillomatosis of rabbits)

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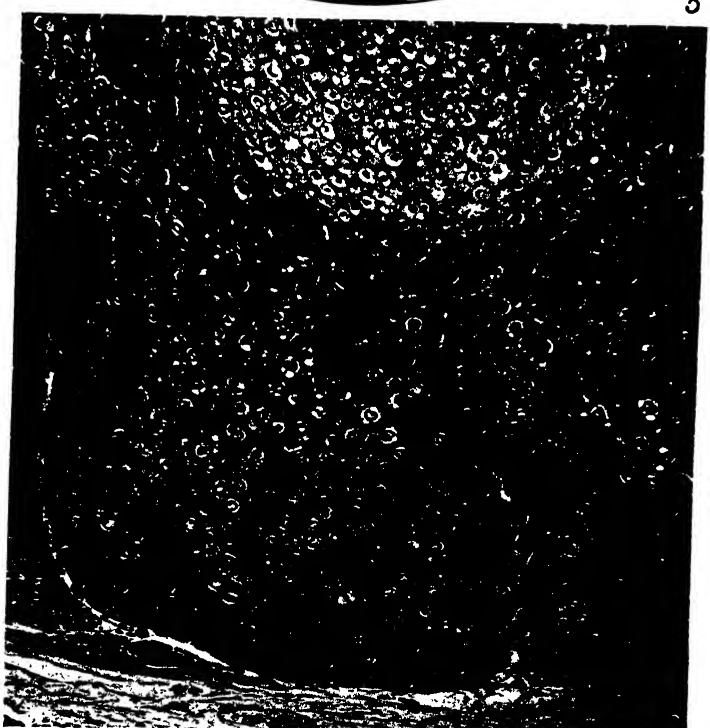

(Shope: Infectious papillomatosis of rabbits)

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(Shope: Infectious papillomatosis of rabbits)

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THE VOLUME OF PRECIPITATE IN PRECIPITIN REACTIONS

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In an attempt to determine the titer of certain precipitins, the end points as determined in the usual way, by titration of a constant quantity of immune serum with the smallest amount of antigen, were too inaccurate for the purpose. Other workers have called attention to certain anomalies in the interpretation of results secured by this method. For example, Manwaring and Azevedo (1) reported that such titrations indicated that the blood serum of a dog injected with horse serum contained about as much horse serum, two weeks after its administration, as a sample of serum drawn within fifteen minutes after administration, since both reacted at the same dilution.

Several methods for recording the activity of precipitins have been devised. The method of optimal proportions of Dean and Webb (2) was of distinct advantage in that it afforded comparisons between serum precipitins. By titration of various sorts they showed that flocculation occurred most rapidly under certain definite conditions regarded as the optimum and for purposes of comparison expressed as the antigen-antibody ratio. They further noted that the zone of most rapid flocculation was not necessarily the point where the maximum quantity of precipitate was formed. Moreover, the method proved unreliable when sera weak in precipitin were employed.

A number of workers considered the quantity of precipitate as a measure of antibody. Nuttall (3) measured the precipitate after two or three days' sedimentation at room temperature. Schur (4) and later Hamburger (5) determined the quantity of precipitate which resulted from mixtures of antigen and antiserum after incubation and centrifugation at high speed. They employed centrifuge tubes to which graduated

capillaries had been fused. More recently Boyden and Baier (6) and Baier (7) have employed thrombocytocrits for quantitative determinations. Baier showed that by using a constant quantity of immune serum and decreasing amounts of antigen the bulk of the precipitate tended to increase within a certain zone of antigenic concentration until a maximum point was reached; beyond this peak the quantity of precipitate rapidly declined. He also considered the effect of temperature, length of incubation, and rate of centrifugation on the quantity of precipitate.

Culbertson (8) in studies of the precipitin reaction showed that accurate determinations of antigen antibody ratio can be made by micro-Kjeldahl analysis of the precipitate. The range where complete binding of both antigen and antibody occurred proved best. The procedure was particularly applicable with crystallized egg albumin and its precipitin. In this way precipitating sera of varying antibody concentrations could be readily compared.

We have already stated our interest in a method for accurate titration of precipitin. A measure of the precipitate seemed to fit our needs. The results obtained by the volumetric method seemed to us to contribute to the knowledge of the problem of specific precipitation, and for this reason seemed worth recording.

EXPERIMENTAL

The sera were prepared by the immunization of rabbits. All were cleared by double centrifugation. The crystallized egg albumin was made by Dr. M. L. Anson of The Rockefeller Institute according to the procedure devised by La Rosa (9). The cow serum used for antigen was clear and had been stored in the refrigerator for several months before use. The crystallized egg albumin was dissolved in salt solution immediately before use. In preparing the antigens the quantity of protein per cubic centimeter was adjusted by means of dilution with 0.9 per cent sodium chloride solution and comparison with a color standard such as employed by Greenberg (10). Antigen of definite protein concentration and immune serum were added to a series of capillary centrifuge tubes. The diameter of capillaries had been calibrated. After mixing the contents the tubes were incubated one hour at 40°C. and then centrifuged at 2850 r.p.m. for three-fourth hour in the maxiforce. The height of the columns was measured by means of the stage micrometer and calibrated ocular under a magnification of 16 diameters. From this the

TABLE 1

Volume of precipitate in cow serum and anti-cow serum

Antigen halved at each dilution. 4 cc. antigen + 0.5 cc. immune serum

TUBE NUMBER	ANTIGEN	VOLUME OF PRECIPITATE
	mgm.	*cu. mm.*
1	80	0.6
2	40	4.05
3	20	5.46
4	10	8.45
5	5	11.03
6	2.5	11.62
7	1.25	7.39
8	0.625	5.46
9	0.312	4.40
10	0.156	3.87
11	0.078	3.17
12	0.039	2.64
13	0.195	1.76
14	0.00975	0.85

TABLE 2

Volume of precipitate in crystallized egg albumin anti-crystallized egg albumin serum

Antigen halved at each dilution. 4 cc. antigen + 0.5 cc. immune serum

TUBE NUMBER	ANTIGEN	VOLUME OF PRECIPITATE
	mgm.	*cu. mm.*
1	40	0.0
2	20	1.06
3	10	1.76
4	5	2.11
5	2.5	2.99
6	1.25	4.40
7	0.625	4.93
8	0.312	6.76
9	0.156	12.85
10	0.078	8.80
11	0.039	5.63
12	0.0195	3.34
13	0.098	1.81
14	0.049	1.06
15	0.0245	0.53

volume in cubic millimeters was calculated. Experiments 1 and 2 are of the same type; experiment 1 is the titration of an anti-cow serum with its specific antigen and experiment 2 is the titration of crystallized egg albumin and anti-crystallized egg albumin. All the essential data are given in tables 1 and 2.

Both sets of data afford definite comparisons and can be considered together. If the data be plotted as in figures 1 and 2,

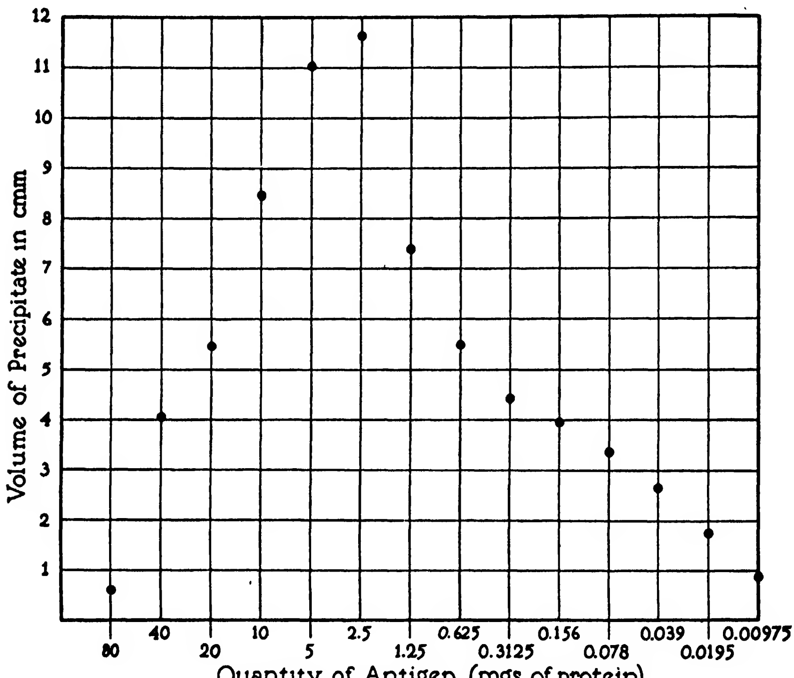

FIG. 1. PLOT OF THE DATA IN TABLE 1

The volume of precipitate resulting from a constant quantity of cow serum precipitin and indicated amounts of antigen.

when the volume of the precipitate is compared with the quantity of antigen, well defined zones are readily determined. At the exterme left, where the antigen is most concentrated, there is relatively little precipitate. This is succeeded by the second zone where, in the case of cow serum anti-cow serum series, there is a marked increase in the quantity of precipitate with each successive halving of the antigen. In the case of the egg albumin

series the rate of increase is slower. The peak in both titrations is clearly defined and can be regarded as the optimum where the ratio of antigen to antiserum is most favorable for precipitation as judged by the quantity of material deposited. In both instances there is a sharp decline in the quantity of precipitate to the right of the optimum.

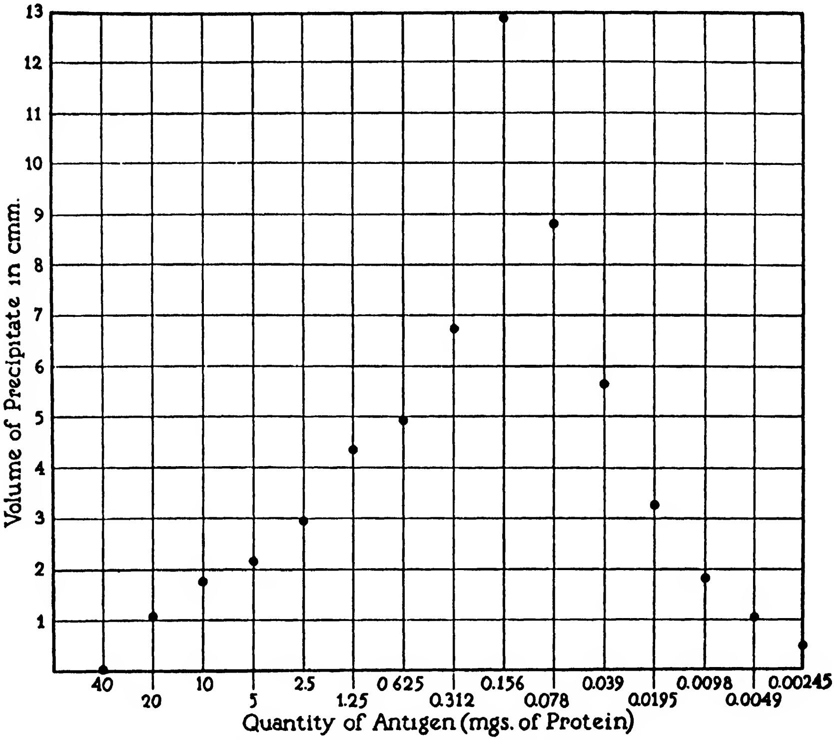

FIG. 2. PLOT OF THE DATA IN TABLE 2

The volume of precipitate resulting from a constant quantity of anti-crystallized egg albumin and indicated amounts of antigen.

The data are not unique in respect to the arrangement of the points indicated in figures 1 and 2, since it has been found that all precipitins tested by this manner behave similarly. An optimum point where the bulk of the precipitate is greatest is always noted by a sharp fall in the quantity of precipitate in either direction as the proportion of antigen is changed.

The points in both graphs suggest a linear arrangement and seem to indicate that within certain zones the quantity of precipitate is the result of the quantitative action of antigen and antibody. The point of maximum effect was of particular interest since it might be expected that at this point both antigen and

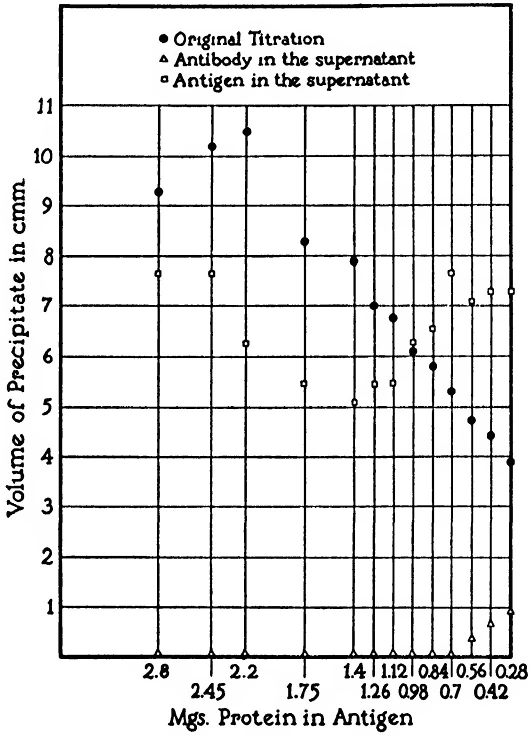

FIG. 3. THE DATA GIVEN IN TABLE 3, COW SERUM AND ANTI-COW SERUM

The volume of precipitate in the region of the zone of minimum precipitation and the effect of changes in quantity of antigen. The data concerning the presence or absence of antigen and antibody in the supernatants after centrifugation are included.

antibody had been completely utilized and thus the supernatant freed of both substances. Our next series of experiments deals with the results of titration of both anti-cow and anti-egg albumin sera in the region of the point of maximum precipitation. In certain instances the quantity of immune serum was varied and in others the quantity of antigen.

In the experiments which follow the quantity of both antigen and antibody left in the supernatant is determined and the results are given in the tables.

Experiment 3. This experiment illustrates the titration of the anti-cow serum when the first tube contains a little more protein than the

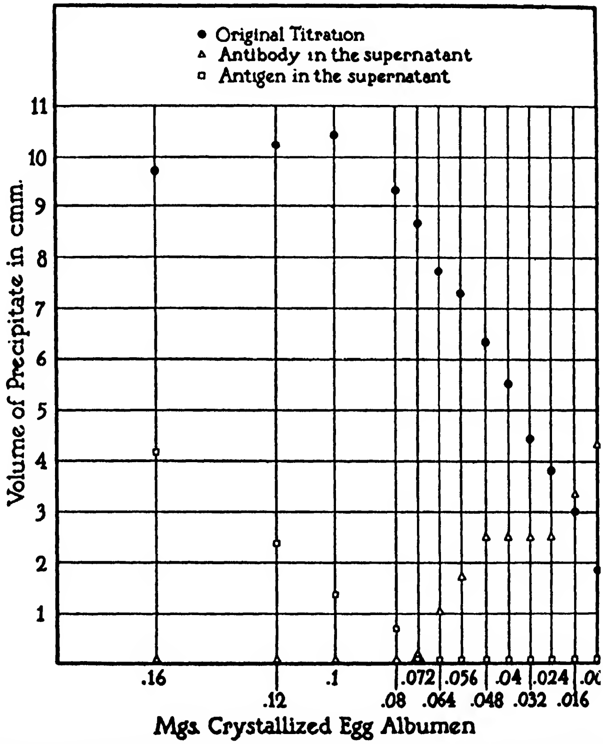

FIG. 4. THE DATA GIVEN IN TABLE 5, CRYSTALLIZED EGG ALBUMIN AND ANTI-CRYSTALIZED EGG ALBUMIN

The volume of the precipitate in the region of the zone of maximum precipitation and the effect of small changes in the quantity of antigen. The data concerning the presence or absence of antigen and antibody in the supernatant after centrifugation are included.

optimum, as determined in table 1. The quantity of immune serum was constant throughout the tubes. The contents of all tubes were uniform, 4.0 cc. antigen, 0.5 cc. immune serum. After the initial precipitaion had been completed and the tubes centrifuged the separate supernatants were withdrawn and were used for the determinations as in parts B and

C. Two cubic centimeters of each supernatant was tested for remaining antibody by the addition of an equal volume of antigen. For determining the quantity of residual antigen in the supernatant, precipitin was added as indicated in the tables and after incubation and centrifugation the volume of the precipitate was found. In the tables the quantity of precipitate determined was calculated to give a value for all the supernatant and this was used for plotting figures 3 and 4.

When the data in table 3 are plotted, as in figure 3, the arrangement of the points to the right of the zone of maximum precipita-

TABLE 3

Titration of anti-cow serum in the zone of maximum precipitation

0.5 cc. anti-cow serum + 4.0 cc. of antigen as indicated

TUBE NUMBER	SERIES A		SERIES B 2 CC. SUPERNATANT A + 2 CC. ANTIGEN (1.4 MGM.)		SERIES C 1.8 CC. SUPERNATANT A 2 CC. IMMUNE SERUM	
	Quantity of protein in antigen	Volume	Obtained volume	Calculated volume	Obtained volume	Calculated volume
	mgm.	*cu. mm.*	*cu mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*
1	2.8	9.33	No precipitate		3 52	7.7
2	2.45	10.2	No precipitate		3.52	7.7
3	2.2	10.56	No precipitate		2.82	6.3
4	1.75	8.27	No precipitate		2.46	5.5
5	1.4	7.92	No precipitate		2.29	5.1
6	1.26	7.04	No precipitate		2.46	5.5
7	1.12	6.86	No precipitate		2.82	5.5
8	0.98	6.16	No precipitate		2.99	6.3
9	0.84	5.81	No precipitate		3.52	6.6
10	0.70	5.28	No precipitate		3.2	7.7
11	0.56	4.75	0.18	0.36	3.3	7.1
12	0.42	4.40	0.35	0.70	3.3	7.3
13	0.28	3.9	0.48	0.96	3.3	.7.3

tion fall approximately in a straight line. Within this zone there is a direct relation between the quantity of antigen and the volume of the precipitate. The maximum effect, as measured by volume of precipitate, cannot be correlated with the complete utilization of either antigen or antibody since, so far as could be determined, all the antibody was utilized long after maximum precipitation occurred. It was not possible to show that in any case all the antigen had been utilized. It appears that the

maximum quantity of antigen has been utilized in about the middle of the series with a rise in its concentration in either direction. Repetition of the experiment always gave the same result—greatest utilization of the antibody from left to right, greatest concentration of antigen at extreme left and right with maximum utilization about midway. Later it will be shown that when similar experiments are done with crystallized egg albumin the results differ markedly.

TABLE 4

Titration of anti-cow serum

Antigen 4 cc. = 2.25 mgm. protein + immune serum as indicated. Total quantity per tube = 4.75 cc.

Tube number	Series A		Series B 2 cc. supernatant of A + 2 cc. antigen		Series C 2 cc. supernatant A + 2.5 cc. antiserum	
	Immune serum	Volume	Obtained volume	Corrected volume	Obtained volume	Corrected volume
	mgm.	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*
1	0.75	11.44	0.53	1.27	2.29	5.35
2	0.5	8.98	0.53	1.27	2.11	5.02
3	0.4	7.04	0.36	0.86	2.46	5.85
4	0.3	4.3	0.36	0.86	3.17	7.54
5	0.25	2.64	0.18	0.43	3.87	9.21
6	0.2	1.58	No precipitate		4.05	9.64
7	0.18	1.32	No precipitate		4.22	10.04
8	0.16	1.06	No precipitate		4.05	9.64
9	0.14	0.88	No precipitate		4.05	9.64
10	0.12	0.7	No precipitate		4.22	10.04
11	0.1	0.53	No precipitate		4.05	9.64
12	0.08	0.35	No precipitate		4.05	9.64
13	0.06	0.24	No precipitate		4.3	10.2

The experiment, however, indicates that beginning at the point of maximum precipitation there is a direct relationship between the quantity of antigen and the amount of precipitate as shown in the primary titration.

In experiment 4 the quantity of antigen remained constant and the amount of immune serum varied. The facts are given in table 4.

The general effect of decreasing the quantity of immune serum is similar to that obtained by reducing the antigen. The quanti-

tative differences are much more marked, as would be expected from the fact that the bulk of the precipitate originates from the immune serum. The arrangement of the quantity of precipitate is linear when plotted. The quantity of precipitate was greatest when the greatest amount of immune serum was used, and least when only 0.06 cc. was employed.

The results from series B indicate that a small amount of antibody remained in the supernatant of the first five tubes only. Antigen remained in all the supernatants throughout the series but the results of the first few readings in series C show that more of the antigen had been bound under these conditions. The same relative values obtained in series C (tubes 6 to 13) suggest that sufficient antigen remained in the supernatants to give maximum precipitation with the unit of antiserum.

It will be noted that no supernatant was completely freed from both antigen and antibody; when the greatest quantities of precipitin were used the supernatant contained a little antigen and a trace of antibody. This was followed by a zone where antibody could not be detected but considerable antigen still remained.

Experiments with crystallized egg albumin and its specific immune serum are clear cut. The incisiveness of the results in experiments 5 and 6, where titrations similar to those given in tables 3 and 4 were made with crystalline egg albumin and its precipitin, afford a contrast to the cow serum precipitin experiments.

Figure 2 illustrates the titration of the crystallized egg albumin serum used in the later experiments. It will be noted that the maximum precipitate was obtained when antigen containing approximately 0.16 mgm. of crystallized egg was mixed with 0.5 cc. of immune serum. This was regarded as the critical point for the further determinations in experiments 5 and 6. The data concerning each experiment are given in the respective tables.

By plotting the data from table 5 in figure 4, the linear arrangement of the points in the primary titration is well defined. As would be expected from the previous observations, the quantity of precipitate to the right of the optimum point declines with each lowering of the antigenic concentration.

The data obtained from the analysis of the supernatants are of interest. Those of tubes 1 to 3 contain easily recognizable quantities of antigen; the supernatant of tube 4 held barely sufficient antigen to produce a precipitate when further precipitin was added. To the right of tube 5 antigen could not be demonstrated. The points are linear in arrangement. The first tube which contained demonstrable antibody was tube 6, and in all tubes to the right of no. 6 antibody was measurable. The linear

TABLE 5

Further titration of crystallized egg albumin antiserum in the zone of maximum precipitation

TUBE NUMBER	SERIES A 4 CC. OF ANTIGEN AS INDICATED BELOW + 0.5 CC. IMMUNE SERUM		SERIES B 2 CC SUPERNATANT A + 2 CC. ANTIGEN		SERIES C 2 CC. SUPERNATANT A + 0.22 CC. IMMUNE SERUM	
	Antigen	Volume	Obtained volume	Corrected volume	Obtained volume	Corrected volume
	mgm.	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*
1	0.16	9.68	No precipitate		2.11	4.22
2	0.12	10.21	No precipitate		1.23	2.46
3	0.10	10.40	No precipitate		0.70	1.40
4	0.08	9.33	No precipitate		0.35	0.70
5	0.072	8.62	No precipitate		No precipitate	
6	0.064	7.74	0.53	1.06	No precipitate	
7	0.056	7.32	0.89	1.78	No precipitate	
8	0.048	6.34	1 23	2.46	No precipitate	
9	0.040	5.46	1.23	2.46	No precipitate	
10	0.032	4.40	1.23	2.46	No precipitate	
11	0.024	3.87	1.23	2.46	No precipitate	
12	0.016	2.99	2.11	4.22	No precipitate	
13	0.008	1.80	2.72	5.44	No precipitate	

arrangement is less exact. Tube 5 is the most critical of the series since neither antigen nor antibody could be demonstrated in the supernatant. It might be said then that 0.072 mgm. of crystallized egg albumin bound all the antibody in 0.5 cc. of immune serum and that all the antigen was utilized in the union.

Experiment 6 records the effect on the volume of precipitate of decreasing quantities of immune serum in a constant quantity of antigen.

The results recorded in experiment 6 are similar to those noted

when cow serum and anti-cow serum were handled in the same way. The maximum precipitate was obtained when the greatest quantity of immune serum was employed. From this point a marked decline in the quantity of precipitate occurred with each diminution of precipitin. The supernatant of all tubes contained antigen, although the low values obtained by adding precipitin in the supernatant indicated that in the tubes containing the greatest quantities of immune serum (series C, 1, 2, 3) considerable crystallized egg albumin had been utilized. Small quantities of antibody could be demonstrated in supernatants of these tubes.

TABLE 6

Final titration of anti-crystallized egg albumin serum

Antigen 4 cc. = 0.16 mgm. crystallized egg albumin. Total content each tube = 4.75 cc.

TUBE NUMBER	SERIES A		SERIES B 2 cc. SUPERNATANT SERIES A + 2 cc. ANTIGEN		SERIES C 2 cc. SUPERNATANT SERIES A + 0.22 cc. IMMUNE SERUM	
	Quantity of immune serum	Volume	Obtained volume	Corrected volume	Obtained volume	Corrected volume
	mgm.	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*	*cu. mm.*
1	0.75	14.43	0.53	1.14	1.23	2.92
2	0.5	9.33	0.35	0.83	2.12	4.9
3	0.4	7.39	Trace		3.34	7.93
4	0.3	4.93	No precipitate		4.4	10.04
5	0.25	3.70	No precipitate		4.58	10.88
6	0.2	3.17	No precipitate		4.93	11.8
7	0.18	2.82	No precipitate		5.36	12.7
8	0.16	2.29	No precipitate		5.36	12.7
9	0.14	1.94	No precipitate		5.63	13.3
10	0.12	1.58	No precipitate		5.63	13.3
11	0.1	1.41	No precipitate		5.36	12.7
12	0.08	1.06	No precipitate		5.36	12.7
13	0.06	0.84	No precipitate		5.63	13.3

DISCUSSION

When the whole titer of a precipitin is considered from the aspect of quantity of precipitate produced by a fixed quantity of antiserum and variable amounts of antigen, there is a sharp parallel between the quantitative findings and the visible effects.

It is well recognized that when too much antigen is present there is little reaction. As the quantity of antigen is reduced the reaction becomes more marked until a maximum is reached and decline begins. It is in this zone that the greatest quantity of precipitate is formed, and by volumetric means this zone has been shown to be relatively narrow in the case of crystallized egg albumin and its precipitin since by the usual titration the quantity of precipitate is distinctly greater in a single tube, as indicated in figure 2. The same is true with cow serum and anti-cow serum but the maximum quantity of precipitate is not so clearly defined, as may be seen from figure 1. When the quantity of antigen was varied only a little from tube to tube much the same general effect, as in figures 3 and 4, was noted with each type of immune serum and antigen. Within relatively narrow limits the addition or subtraction of minute quantities of antigen failed to change the quantity of precipitate. Once, however, the optimum was reached, diminution of antigen even as little as 0.03 mgm., as indicated in figure 4, produced a marked effect on the quantity of precipitate.

The volume of precipitate may be correlated directly with the quantity of antigen or antibody provided the optimum zone, as indicated by the greatest quantity of precipitate, is chosen for the experiments. It is true that the most regular effect on precipitate volume results when antigen is diminished; when the data from such experiments are plotted the linear arrangement of the points is striking. When the antigen was kept constant and the quantity of antiserum varied the changes in the bulk of the precipitate were most marked at the two extremes; for example 0.75 cc. of anti-egg albumin serum produced 14.2 cu. mm. precipitate while 0.06 cc. of the same serum produced only 0.8 cu. mm.

When the supernatant fluids from the various experiments were tested volumetrically for antigen and antibody differences between the two types of precipitin were readily manifested. With crystallized egg albumin and its immune serum it is possible to demonstrate under proper conditions, as in figure 4, that antigen is completely utilized beyond a definite level. It happened that the first point where no antigen was found was the

last point from the left where antibody was absent. Culbertson's determinations indicated that at the point of neutralization the precipitate comprised an antigen-antiserum ratio of 1:13. Therefore, the precipitate might be expected to comprise 0.072 mgm. egg albumin and 0.94 mgm. of serum protein in the tube where both substances were bound. The titration of the residual antibody shows essentially the same graphic features except that the maximum, as determined quantitatively, is to the right. The two substances, as determined volumetrically, are as two sides of a right angle, the apex at the point of complete binding on the base line and the quantities of precipitate resulting from the addition of new antigen and precipitin as the sides of the angle. It is of interest to note that the point where the greatest quantity of precipitate was obtained was not the point where both antigen and antibody have been completely bound. This is illustrated in figure 4 and table 5.

The preceding applies only to relatively pure antigens such as crystallized egg albumin and its precipitin. Cow serum and anti-cow serum and, from the results of Culbertson, horse serum and anti-horse serum behave differently. In the experiments with anti-cow serum and its antigen, as in figure 3, essentially the same linear result was obtained but the determinations on the supernatants were markedly different. In no case have we been able to show that all the antigen was combined with all the antibody to produce complete binding of both. In extreme cases, as illustrated in the last three points in figure 3, the supernatants contained both antigen and antibody. The argument advanced by Culbertson, who obtained the same results with horse serum and its precipitin, that horse serum is composed of more than one antigen and presumably equivalent precipitins, is strengthened by our experimental data with cow serum and its precipitins. Multiplicity of antigens and antibodies might explain the peculiar type of reaction in experiment 3. The experiment indicated that the tubes which originally contained the most antigen still had considerable antigen in the supernatant after completion of the reaction. The second titrations indicated that as the original antigenic concentration decreased, the amount of antigenic cow

serum in the supernatant was distinctly lessened until a minimum was reached when the amount of precipitate resulting from the introduction of more precipitin increased at an even rate. Thus it might be assumed that the first precipitation absorbed a considerable amount of one type of antigen and much less of others and that these unabsorbed antigens were responsible for the secondary increase in the quantity of precipitate when new precipitin was added. The titration of residual antibody in the same experiment seems to fit into this view since for practical purposes even as little as 0.56 mgm. of cow serum protein bound over 95 per cent of the antibody.

SUMMARY

A procedure has been described whereby the quantity of precipitate resulting from the action of precipitin and antigen can be measured. At one point in titrations, as usually practiced, the greatest quantity of precipitate is formed and this we have called the zone of maximum precipitation. With this as a basis we have been able to show that the quantity of precipitate will vary proportionately with change in quantity of either antigen or immune serum. A linear type of reaction has been demonstrated. In addition similar linear relationships have been shown in the determinations of antigen and antibody in the supernatant after precipitation has taken place. In cow serum and its precipitin we have not been able to show that in a given series either antigen or antibody is completely utilized. Where a single antigen, such as egg albumin, was employed with its precipitin, there occurred within a narrow zone complete binding of both antigen and antibody, while in either direction one or the other was still present in active form.

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CULTIVATION OF PSEUDORABIES VIRUS

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PLATE 40

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All attempts at the cultivation of pseudorabies virus reported in the literature have been unsuccessful. Aujeszky (1) who first described the disease in 1902, Zwick and Zeller (2), Schmiedhoffer (3), Bertarelli and Melli (4), and others were unable to grow the causative agent on lifeless media aerobically or anaerobically. Sangiorgi (5) attempted to cultivate pseudorabies virus by the method devised by Flexner and Noguchi (6) for the cultivation of the globoid bodies in poliomyelitis. The first series of Sangiorgi's cultures was still infectious after 8 days' incubation at 37°C. The first series of subcultures, however, was inactive after 15 days' incubation.

Since pseudorabies virus in all its qualities is a typical representative of the filtrable viruses, some of which have been successfully cultivated in the presence of living cells, an attempt was made to grow it in this manner.

Pathogenic Properties of the Virus Studied

The Hungarian strain of pseudorabies virus was used in our experiments. In 1930 a sample of this strain had been sent to Dr. Shope by Prof. A. Aujeszky of Budapest. Since then the virus has been maintained by rabbit passage and storage in glycerol.

When injected subcutaneously into rabbits and guinea pigs pseudorabies virus causes a regularly fatal disease. Rabbits are considerably more susceptible than guinea pigs. In both species a characteristic symptom constantly present after subcutaneous infection is pruritus, which leads to repeated biting, scratching, and consequent mutilation of the skin at the site of inoculation. The incubation period is from 2 to 3 days and death usually occurs in from 12 to 24 hours after the onset of the first symptoms. More detailed descriptions of the clinical picture in pseudorabies are given by Aujeszky (1), Zwick and Zeller (2), Schmiedhoffer (3), and Shope (7).

While all authors have found both rabbits and guinea pigs susceptible to pseudorabies, their findings in regard to its pathogenicity for mice have been at variance.

Aujeszky (1) found mice to be less susceptible to the virus than rabbits, dogs, and guinea pigs. Schmiedhoffer (3) calls gray mice more susceptible than white ones. Von Râtz (8) could infect white mice by feeding the virus. The incubation period after this mode of infection was very long, 6 to 15 days. In Isobolinski and Patzewitsch's (9) experiments mice were susceptible to pseudorabies. The incubation period in white mice was 3 to 4 days. Zwick and Zeller (2) could not infect white mice by subcutaneous, intramuscular, or intraperitoneal injections of virus. In Shope's (7) experiments with the Iowa ("mad itch") strain of pseudorabies virus white mice developed fatal infections regularly from intracerebral inoculation, irregularly from intraperitoneal infection, and never from subcutaneous injections. Burggraaf and Lourens (10), using pseudorabies virus from an outbreak in Holland in 1932, could not infect mice by intraperitoneal or subcutaneous injection. Unless there are variations in the susceptibility of white mice used by different laboratories to pseudorabies virus the different results obtained by the authors mentioned above, who worked largely with the Hungarian strain, cannot be explained.

Since it was intended to use white mice for the titration of the virus, it was necessary to know whether our mice were regularly susceptible to the Hungarian strain of pseudorabies virus or not.

In a preliminary experiment it was found that white mice die regularly in 3 to 4 days after a sufficiently large dose of virus administered intraperitoneally. The intraperitoneal route of infection was preferred to the intracerebral one, since a greater amount of fluid could be injected in this way and therefore the dosage could be regulated more accurately.

The disease in mice was found to be as constantly fatal as in guinea pigs and rabbits. When approximately 9/10 of the inoculum was injected intraperitoneally and 1/10 subcutaneously at the same site, 70 to 80 per cent of the mice showed the symptoms characteristic in rabbits and guinea pigs after subcutaneous injection: active biting and scratching, leading to self-mutilation at the site of inoculation or some other place on the body surface. The mice which failed to develop pruritus showed a greatly accelerated respiratory rate, dyspnea, and salivation. After some experience with the disease in mice it is not difficult to establish the clinical diagnosis in every case.

In Table I the results of a titration of rabbit brain pseudorabies

virus in mice are given. For comparison, four guinea pigs were injected with decimal dilutions of the same virus. Both guinea pigs and mice were killed regularly.

TABLE I

Titration of Rabbit Brain Pseudorabies Virus

Dose* (virulent rabbit brain)	White mice (intraperitoneally)		Guinea pigs (subcutaneously)	
	No.	Result	No.	Result
mg.				
100	1	Died in 66 hrs.	778	Died in 73 hrs.
	2	" " 74 "		
50	3	" " 66 "		
	4	" " 114 "		
10	5	" " 66 "	777	Died in 82 hrs.
	6	" " 74 "		
	7	" " 68 "		
	8	" " 75 "		
5	9	" " 81 "		
	10	" " 125 "		
2.5	11	" " 73 "		
	12	" " 92 "		
1	13	No illness	776	Died in 91 hrs.
	14	" "		
0.1	15	" "	775	No illness
0.01	16	" "		

* Each dilution administered in 1 cc. of physiological saline.

Cultivation in Rabbit Testicle Media

Minced rabbit testicle was chosen as the tissue constituent of the culture media, since among animals the rabbit is most susceptible to pseudorabies virus and since the virus can apparently multiply in the testicle *in vivo*. Shope (7) found the testicles to be a dependable source of virus after intratesticular inoculation. Moreover, it is known that vaccinia virus (Maitland and Laing (11)), Virus III

(Andrewes (12, 13)), and herpes virus (Andrewes (14)) can be cultivated *in vitro* in series in minced rabbit testicle suspended in rabbit serum and Tyrode solution.

Preparation of Media Used

The testes of a healthy adult rabbit were removed aseptically, washed twice in physiological salt solution, and finely minced with long scissors on a watch-glass contained in a Petri dish. The tissue pulp in amounts of 100 to 150 mg. was distributed with a large loop or by wide mouth pipettes to 50 cc. Florence flasks. The flasks were closed with cotton plugs covered with a layer of tin-foil. As a rule, 2 cc. of rabbit serum (mixtures of sera from several normal rabbits were used) and 2 cc. of Tyrode solution prepared according to the formula given by Fischer (15) were added at once to the tissue pulp. The flasks were then slightly shaken to distribute the tissue fragments equally in the fluid. Sterilization of the serum and Tyrode solution had been effected by passage through Berkefeld N or W filters. The cultures of each serial passage were made up in duplicate or triplicate. From the testes of every rabbit killed for tissue, enough media for two or three culture series was usually made up. Uninoculated culture flasks were stored in the refrigerator, at approximately +4°C., until needed.

Course of the Serial Cultivation

Each flask of Culture Series I was inoculated with 0.3 cc. of a 10 per cent brain emulsion from a rabbit that had died of pseudorabies following intracerebral inoculation. Subcultures were inoculated with 0.4 cc. of the cultures of the preceding series. Thus the dilution factor in the serial culture passages was about 11. The inoculum usually contained a number of small tissue fragments which were drawn into the pipette with the fluid. After inoculation the flasks were either put into the refrigerator for 1 hour according to the method of Carrel (16), or directly into the incubator after it had been found that storage in the refrigerator prior to incubation was not necessary. This observation agrees with Andrewes' (13) findings concerning Virus III. Incubation was carried out at 37°C. for 2 days unless otherwise stated. After incubation the cultures were tested for sterility and contaminated cultures were discarded. The presence of the virus in cultures was determined by subcutaneous injection of 1 cc. of cell-containing culture fluid into a guinea pig or a rabbit.

The titer of the cultures was determined by injecting decimal dilutions of whole ground cultures or culture fractions into white mice. Before the titration of whole cultures the fluid and tissue were carefully separated. The tissue fragments were ground with a small amount of sand in a mortar and then the fluid portion slowly added. From the suspension thus obtained decimal dilutions were made with physiological salt solution. A fresh pipette was used for every dilution, 1 cc. of which was injected into one or two mice, 9/10 of the dose intraperitoneally, 1/10

subcutaneously at the same site. When, for comparison, the titer of the fluid and tissue portions of cultures was determined, they were centrifuged, following which the supernatant fluid was pipetted off and divided into two equal parts. The tissue was ground with sand and one-half of the supernatant fluid was added to obtain a suspension which could be injected. Decimal dilutions of this suspension and of the other half of the supernatant fluid were tested in mice. As a rule, several cultures of a serial passage were used for titration to lessen the effect of variations in virulence of single cultures. The results of the cultivations are given in Table II.

The virus was cultivated uninterruptedly for 12 serial passages. The cultures of Series XIII were inactive when tested in mice. In this series media were used which had been stored in the refrigerator for 6 days prior to inoculation. It is likely that the cells in the medium had died during this time and were no longer suitable for the multiplication of virus.

To determine whether the virus had actually disappeared from the cultures or whether its pathogenic properties for mice had been altered, subcultures were made and tested in guinea pigs and rabbits by subcutaneous and intracerebral inoculation. These animals developed no illness. The guinea pigs, when tested for immunity 3 weeks later, succumbed to the disease.

A new set of cultures (Xa) was inoculated from the cultures of Series IX which had been stored in the refrigerator for 21 days after incubation and proven to be still virulent for mice. The virus was then carried through 20 further serial culture passages. The cultures of Series XXXI were contaminated by bacteria and avirulent for mice. Therefore a new series of cultures (XXIXa) was started from Series XXVIII, the cultures of which had been kept in the refrigerator for 5 days. After the virus had been cultivated in 4 more serial passages the experiment was discontinued.

The titer of the serial cultures fluctuated considerably, as Table I shows. The reason for this fluctuation is not known. It is noteworthy that, while 1 cc. of the cultures of Series XXIX failed to infect a guinea pig by subcutaneous inoculation, 0.4 cc. of the same cultures contained enough virus to infect subcultures. The tissue portion of the cultures of Series XXV reached the titer of at least 1:1 million, which is almost incredibly high for pseudorabies. In all cultures fractionally titrated the tissue portion contained consid-

TABLE II

Cultivation of Pseudorabies Virus in Rabbit Testicle Medium

No. of serial culture passage	Date	Test for virulence by subcutaneous inoculation				Titer* of culture virus for mice			Length of time media was stored in refrigerator before inoculation
		Rabbits		Guinea pigs		Fluid portion of cultures	Tissue portion of cultures	Whole ground cultures	
		No.	Result	No.	Result				
	1933								*days*
I	Feb. 15	•†	•	•	•	1:10	•	•	0
III	" 19	574	+ 56 hrs.	•	•	•	•	•	0
V	" 28	652	+ 71 "	769	+ 55 hrs.	•	•	•	0
VIII	Mar. 6	•	•	768	+ 70 "	•	•	1:100	2
IX	" 8	•	•	789	+ 62 "	•	•	•	4
XII	" 14	•	•	805	+ 95 "	•	•	•	4
XIII	" 16	•	•	•	•	•	•	Avirulent	6
Xa (inoculated with 0.4 cc. of IX)	Apr. 1	•	•	821	+ 61 hrs.	•	•	•	0
XIIa	" 5	•	•	820	+ 64 "	•	•	1:1,000	4
XIIIa	" 7	•	•	828	+ 64 "	•	•	1:1,000	0
XIIIa	" 7	•	•	834	+ 67 "	•	•	1:1,000	0
XIV	" 9	•	•	822	+ 64 "	•	•	1:1,000	2
XIV	" 9	•	•	826	+ 67 "	•	•	1:1,000	2
XVI	" 13	•	•	855	+ 72 "	•	•	1:1,000	0
XX	" 21	•	•	841	+ 60 "	1:100	1:10,000	•	2
XXI	" 23	•	•	860	+ 92 "	•	•	Avirulent	4
XXIV	" 29	•	•	876	+ 46 "			1:10,000	0
XXV	May 1	•	•	839	+ 67 "	1:100	1:1 million	•	2
XXVII	" 5	•	•	881	+ 57 "	Avirulent	1:100	•	0
XXVIII	" 7	•	•	883	+ 66 "	•	•	•	2

XXIX	"	10	•	•	884	—	•	•	Avirulent	5
XXX	"	12	•	•	891	+ 99 hrs.	•	•	•	0
XXXI (contaminated)	"	14	•	•	•	•	•	•	Avirulent‡	2
XXIXa (inoculated with 0.4 cc. of XXVIII)	"	14	•	•	895	+ 70 hrs.	•	•	•	2
XXXa	"	16	•	•	889	+ 57 "	•	•	•	0
XXXIa	"	18	•	•	•	•	Avirulent	•	•	0
XXXII	"	20	•	•	•	•	•	•	1:10	4

* By titer in this table and the following ones is meant the highest decimal dilution, 1 cc. of which killed mice when inoculated 9/10 intraperitoneally and 1/10 subcutaneously.

† • = not tested; + = died after; — = no illness.

erably more virus than the fluid portion (see Series XX, XXV, XXVII, and XXXIa in Table II). The tissue could not be freed from virus by repeated washing.

In Table III the results of the titration of the cultures of the last series with rabbit testicle tissue are given as an example of the titration of cultivated virus.

Various factors governing the growth of the pseudorabies virus in cultures were studied in the following experiments.

TABLE III

Titration of Cultures of Series XXXII in Mice

Mouse No. (Series 33)	Dose (ground culture suspension)	Result
	cc.	
1	1.0	Died in 79 hrs.
2	0.1	" " 90 "
3	0.01	" " 104 "
4	0.01	No illness
5	0.001	" "
6	0.001	" "
7	0.0001	" "
8	0.0001	" "

Amount of Tissue Required

Rivers (17)[1] and Rivers and Ward (18) observed that too large amounts of tissue in cultures of vaccinia virus inhibited or prevented the multiplication of the virus. For pseudorabies virus the same seems to be true, although this virus requires a somewhat greater amount of tissue than does vaccinia virus. In Experiment 4 (Table IV) the virus did not multiply in Culture 1 containing 805 mg. tissue, whereas the titer of the control culture (135 mg. tissue) inoculated from the same source was at least 1:1,000. In cultures which contained from 12 to 270 mg. tissue, and which were inoculated with the usual amount of culture material, the virus readily multiplied. A

[1] According to a personal communication from Dr. Rivers, there is a misprint in his paper on "Cultivation of vaccine virus for Jennerian prophylaxis in man," *J. Exp. Med.*, 1931, **54**, 454, line 30: instead of reading "approximately 1 gm. of minced chick embryo tissue," the passage should read "approximately 0.1 gm. . ."

relationship seems to exist between the amount of tissue in a culture and the amount of inoculum used in infecting it: the smaller the amount of inoculum, the greater the amount of tissue required in the culture to insure infection (Experiments 1 and 2, Table IV).

Amount of Serum Required

That rabbit testicle culture medium must contain serum in order to support growth of pseudorabies virus was shown by three unsuccessful attempts to grow the virus in rabbit testicle tissue suspended in Tyrode solution without rabbit serum, according to the method of Li and Rivers (19).

TABLE IV

Influence of the Amount of Tissue in Cultures on the Multiplication of Pseudorabies Virus

Experiment No.	Inoculum cc.	Inoculum Culture passage	Culture No.	Amount of tissue	Titer for mice
				mg.	
1	0.1	IX	1	130	1:100
			2	50	Avirulent
			3	10	"
2	0.1	XIIIa	1	270	<1:1,000
			2	140	1:100
			3	10	1:10
	0.4	XIIIa	4	210	1:1,000
			5	82	1:1,000
			6	12	1:1,000
3	0.4	XIa	1	180	1:1,000
			2	90	1:100
			3	10	1:100
4	0.4	XV	1	805	Avirulent
			2	135	<1:1,000

In the first experiment a medium consisting of 100–150 mg. minced rabbit testicle suspended in 4 cc. Tyrode solution was inoculated with 0.5 cc. of a 10 per cent suspension of virulent rabbit brain. After the cultures had been incubated for 3 days at 37°C. they were avirulent for guinea pigs.

In the second experiment culture virus[2] was used as inoculum for the 1st series of cultures. The virus was still demonstrable in the 2nd series of subcultures. The 4th, 5th, and 6th series of subcultures were avirulent. The virus could apparently multiply in the cultures of the first 2 series because they contained traces of serum from the inoculum. When the amount of serum introduced in this way had reached a certain dilution, multiplication of the virus was no longer possible. In control cultures containing 2 cc. rabbit serum and 2 cc. Tyrode solution the virus readily multiplied.

TABLE V

Influence of the Ratio of Rabbit Serum to Tyrode Solution in Cultures on the Multiplication of Pseudorabies Virus

Culture series	Ratio of serum to Tyrode solution	Inoculum		Test for virulence and titer of serial passages			
		cc.	Culture passage	1st passage	2nd passage	3rd passage	4th passage
a	1:1	0.4	XX	•*	•	•	<1:1,000
b	1:3	0.4	XX	1 cc. s.c. Guinea Pig 870. Died in 58 hrs.	1 cc. i.p. in mouse. Died in 51 hrs.	•	<1:1,000
c	1:9	0.4	XX	•	•	•	1:100
d	1:49	0.4	XX	•	•	•	1:100
e	1:99	0.4	XXV	1 cc. i.p. in 2 mice. Died in 92 and 112 hrs.	•	1 cc. i.p. in 2 mice. No illness	
f	1:999	0.4	XXV	1 cc. i.p. in mouse. Died in 68 hrs.	•	1 cc. i.p. in 2 mice. No illness	
g (control for e and f)	1:3	0.4	XXV	•	•	1 cc. i.p. in mouse. Died in 93 hrs.	

* • = not tested; s.c. = subcutaneously; i.p. = intraperitoneally.

[2] By "culture virus" is meant pseudorabies virus that has been grown *in vitro* by the methods of cultivation described. The term "culture virus" is used in distinction to the term "brain virus" by which is meant pseudorabies virus from the brain of an animal dead following cerebral infection.

The third experiment was carried out similarly. The 1st series of subcultures was still virulent; the 2nd series, however, was avirulent.

An attempt was then made to determine the ratio of serum to Tyrode solution most favorable to the multiplication of the virus. The results of these experiments are recorded in Table V.

The virus multiplied best in cultures in which the ratio of serum to Tyrode solution was 1:1 and 1:3. In cultures in which this ratio was 1:99 and 1:999, the virus could not be cultivated in series, although it multiplied in serial control cultures containing 1 part serum to 3 parts Tyrode solution inoculated from the same source. During the serial cultivation of pseudorabies virus the impression was gained that 1:1 represented a more favorable proportion of serum to Tyrode solution than 1:3.

TABLE VI

Influence of Time of Incubation of Cultures on Multiplication of Pseudorabies Virus

Experiment No.	Inoculum *cc.*	Inoculum Culture passage	Length of time incubated	Titer for mice
			days	
1	0.4	XIIa	2	1:1,000
			3	1:100
			4	1:100
2	0.4	XV	2	1:1,000
			3	1:1,000
			4	Avirulent
			5	"
			6	"
3	0.4	XXIII	1	1:10
			2	1:100
			3	1:1,000
			4	Avirulent
			5	"
			6	"

Time of Incubation

A number of cultures containing equal amounts of fresh tissue, 2 cc. rabbit serum, and 2 cc. Tyrode solution were inoculated from the same source and titrated in mice after various periods of incubation at 37°C. The results are shown in Table VI.

In Experiments 1 and 2 (Table VI) the cultures had reached their maximum virus content after 2 days' incubation and in Experiment 3 after 3 days' incubation. Incubation of from 4 to 6 days inactivated the cultures in Experiments 2 and 3; this was probably due, as is shown later, to the necrosis of the tissue. For the cultivation of the virus in series 2 days' incubation proved to be sufficient.

Cultivation in Long Test-Tubes

Maitland, Laing, and Lyth (20) found that while vaccinia virus could be grown in Carrel flasks in which the depth of the medium was from 1 to 2 mm., it did not multiply in test-tubes when the height of the column of the fluid covering the tissue was 15 mm. Under such conditions the respiration of the tissue, as measured by the method of Warburg, ceased after 24 hours' incubation at 37°C. The authors believed that there was a correlation between the multiplication of the virus and the respiratory activity of the cells contained in the medium.

The growth requirements of pseudorabies virus differ from vaccinia virus since multiplication was demonstrated in 100–150 mg. minced rabbit testicle tissue suspended in 3 cc. Tyrode solution and 1 cc. rabbit serum in long test (Noguchi) tubes, in which the column of fluid overlying the tissue was from 5–6 cm. high.

Length of Time Media May Be Stored in Refrigerator before Inoculation

The question of how long minced rabbit testicle tissue suspended in 2 cc. rabbit serum and 2 cc. Tyrode solution could be stored in the refrigerator before inoculation was of importance in these experiments. In almost every case the tissue was still suitable for the multiplication of virus after it had been stored in the refrigerator for 4 days prior to inoculation. In one case (see Culture Series XXIX, Table I) the virus did not multiply in tissue which had been kept in the refrigerator for 5 days. In Culture Passage XIII a medium was used which had been stored in the refrigerator for 6 days. The virus became inactive in this medium after 2 days' incubation. In four other experiments media stored in the refrigerator for 6 days were tested. In two of these cases the virus was no longer demonstrable after 2 days' incubation, in the other two cases there was slight multiplication of the virus.

In culture media which had been in the refrigerator for 7, 8, 9, and 10 days prior to inoculation with virus, the virus did not multiply. Such cultures were inactive after 2 days' incubation. In all these experiments the virus multiplied in control media which were fresh or had been kept in the refrigerator for from 1 to 4 days. The failure to grow in stored media is probably due to death of the tissue.

Time of Survival of Culture Virus in the Refrigerator

Cultures of known virulence were stored in the refrigerator for various periods of time and then again tested for their virulence for mice. All cultures were still active after they had been kept in the refrigerator for from 1 to 15 days; after 19 days one culture out of two was avirulent; after 21 days three out of three cultures were still virulent. All cultures which had been in the refrigerator longer than 21 days were inactive. Subcultures could be made from cultures which had been stored in the refrigerator for 1, 2, 4, 5, 16, and 21 days.

Histological Examination of Culture Tissue

Freshly prepared culture media (100–150 mg. minced rabbit testicle suspended in 2 cc. rabbit serum and 2 cc. Tyrode solution) were inoculated with culture virus as usual and incubated for 1, 2, 3, or 4 days. As controls, uninoculated media from the same source were incubated simultaneously for the same period of time. After incubation, pieces of tissue from the culture and control flasks were fixed in Zenker's or Allen's fluid. Paraffin sections were made and stained with phloxin-methylene blue or hematoxylin-eosin. Mouse inoculations in every case showed that virus was present in those cultures from which pieces of tissue had been removed.

Sections from cultures incubated for 1 day showed slight necrosis of the epithelium and very little necrosis of the interstitium. In some interstitial cells the nuclear membranes were slightly hyperchromatic. No inclusions were seen.

In sections from cultures incubated for 2 days necrosis was more pronounced. The interstitium still looked fairly healthy, especially at the edge of the sections. Acidophilic intranuclear inclusions appeared in the interstitial cells of Leydig, in endothelial cells of capillaries, and in connective tissue cells of the lamellar membranes of the tubules (Fig. 1). These inclusions resembled those described by Hurst (21) in many different kinds of cells of rabbits infected with pseudorabies. They were irregular in size, and, sometimes, in shape. Some-

times they had well defined margins. Some nuclei contained several small inclusions, others a single large one. In inclusion-bearing cells and many others hyperchromatosis of the nuclear walls was constantly present; such hyperchromatosis appeared also in sections from uninoculated controls, but in them inclusions were never seen. When the inclusions were completely formed, there was a narrow free space between them and the nuclear membrane. In cells of the seminiferous epithelium inclusions were never found.

In cultures incubated for 3 days there was considerably more necrosis of the tissue. Inclusions were still present.

The tissue of cultures incubated for 4 days was generally necrotic. The nuclei of the cells in which inclusions usually appeared were karyorrhectic. Inclusions were no longer distinct (Fig. 2). In sections from uninoculated control media the interstitium, after 4 days' incubation, still looked surprisingly healthy (Fig. 3). The fact that tissues from cultures were always much more necrotic than those from uninoculated controls is attributed to the action of the virus.

Intranuclear inclusions appeared in only about 60 per cent of the pseudorabies cultures examined histologically. The reason for this irregularity may be that, in some cultures, not all of the many small pieces of tissue become infected by the virus, and that uninfected tissue particles were picked out in those cases in which inclusions were not found.

Inclusions of the same type and in the same kind of cells were also found *in vivo* in the testis of a rabbit that had been infected intratesticularly with virus from rabbit brain. The inclusions were completely formed 24 hours after inoculation, that is, during the incubation period.

Many of the inclusion bodies in pseudorabies cultures are somewhat similar to those found by Andrewes (12) in rabbit testicle cultures of Virus III. The Virus III inclusions, however, appeared in interstitial cells exclusively.

Attempts to Cultivate Pseudorabies Virus in Rabbit Kidney, Liver, and Blood

Since vaccinia virus was found by Maitland and Laing (11) and others to multiply in minced rabbit kidney tissue suspended in rabbit

serum and Tyrode solution, four attempts were made to grow pseudorabies virus under similar conditions. In two of these experiments, cell-free extracts of rabbit brain and rabbit testicle were added to the medium containing approximately 100 mg. minced rabbit kidney in 2 cc. rabbit serum and 2 cc. Tyrode solution. The same technique was followed as for the cultivation of the virus in testicular tissue. In none of the four experiments did pseudorabies virus multiply.

Two experiments with minced rabbit liver and one experiment with rabbit blood were equally unsuccessful.

It may be recalled at this time that Andrewes (13) was unable to grow Virus III in media with minced rabbit kidney and liver as tissue constituents.

Cultivation of Pseudorabies Virus in Guinea Pig Testicle Media

After several unsuccessful attempts pseudorabies virus was cultivated in series in minced testicle tissue taken from guinea pigs of about 500 gm. in weight and suspended in 1 cc. guinea pig serum + 3 cc. Tyrode solution. This ratio of serum to Tyrode solution appeared to be favorable to the multiplication of the virus. Virus cultivated in chick embryo media (see below) was used to infect the cultures of the first series. After the 6th serial passage of the virus through the guinea pig medium the experiment was discontinued. When rabbit serum was substituted for guinea pig serum in this medium, the virus failed to multiply. The pathogenic properties of pseudorabies virus cultivated in guinea pig testicle medium did not differ from that in rabbit testicle medium. The possibility of cultivating pseudorabies virus in guinea pig testicle tissue will be made use of later, when an attempt will be made to grow the virus in tissue from guinea pigs immune to the disease.

Cultivation of Pseudorabies Virus in Chick Embryo Media

Ten day chick embryos were finely minced with scissors, after their eyes had been removed, and 100–150 mg. of the tissue pulp was suspended in 3 cc. Tyrode solution and 1 cc. sheep serum. The same technique was followed as for the cultivation of the virus in rabbit testicle tissue. Virus from the 30th culture series in rabbit testicle medium was used to start the cultures, and the results of the experi-

ment, given in Table VII, show that pseudorabies virus multiplied in the chick embryo media.

From the 5th passage in chick embryo medium the virus was cultivated in both serum-containing (A) and in serum-free (B) media. The medium of Group A was the same as used in the previous culture series; while the medium of Group B, 100–150 mg. of minced chick embryo tissue suspended in 4 cc. Tyrode solution, was the same as used by Li and Rivers (19) for the cultivation of vaccinia virus.

TABLE VII

Cultivation of Pseudorabies Virus in Chick Embryo Tissue

No. of serial culture passage*	Test for virulence		Titer for mice
	Guinea pigs (subcutaneously)	Mice (intraperitoneally)	
Ch. E. I	•†	+	•
" II	+	•	•
" III	+	•	•
" IV	•	+	1:100
" V A	•	+	•
" V B	•	+	•
" VII A	+	•	•
" VII B	+	+	•
" X A	•	+	1:10
" X B	•	+	1:100
" XII A	+	•	•
" XII B	+	•	•
" XIII A	•	+	1:1
" XIII B	•	+	1:100
" XVI B‡	+	•	•
" XIX B	•	+	•

* Started from Transfer XXX in rabbit testicle medium.
† • = not tested; + = virulent.
‡ Series A discontinued.

In the rabbit and guinea pig testicle media a certain amount of serum had been necessary to insure multiplication of pseudorabies virus, but in chick embryo cultures serum was not necessary. The growth of the virus was even better in cultures which did not contain serum (Table VII). The titer of the chick embryo cultures, on the average, was considerably lower than that of the rabbit testicle cultures.

After Culture Series Ch. E. XIII the cultivation in media containing serum was discontinued. In serum-free media the virus was still under cultivation when this paper was completed.

Properties of Culture Virus

Pseudorabies virus did not change its pathogenic properties for mice, guinea pigs, and rabbits in the course of the cultivation and consistently induced a fatal disease, the salient feature of which was violent pruritus causing self-mutilation. The disease following the injection of cultivated virus had a shorter incubation period, a more rapid course, and the lesions at the site of inoculation were larger than in control animals receiving the brain passage virus.

Serum from guinea pigs immunized against either the Hungarian or Iowa strain of brain passage pseudorabies virus neutralized culture virus when the mixtures of serum and virus were tested in the usual way by subcutaneous inoculation into guinea pigs. The culture virus used in the neutralization tests had been under continuous cultivation for 36 serial passages, thus indicating that no immunological change had taken place; that is, it was still immunologically identical with the brain virus from which the cultures were originally made.

In like manner culture virus maintained its filtrability after prolonged cultivation outside the animal body, as was evidenced by the readiness with which culture virus of the 39th serial passage passed Berkefeld filters of N porosity.

Thus, in none of its salient properties did culture virus differ from the brain virus used in initiating the cultures; it was still filtrable, it was still neutralized by virucidal sera prepared against brain virus, and it still produced the typical clinical and pathological picture of pseudorabies in experimental animals.

DISCUSSION

Like many other filtrable viruses, pseudorabies virus can be cultivated in series in the presence of living tissue. The fact that the virus failed to multiply in tissue which had been stored in the refrigerator for more than 6 days prior to inoculation with virus, and in cell-free 20 per cent rabbit testicle extract suggests that living cells are necessary for its growth.

Growth has been obtained in media containing rabbit or guinea pig testicle or chick embryo tissues. Rabbit testicle tissue gave the best results both as to the regularity of the multiplication of the virus and the concentration the virus attained in the cultures. The chick em-

bryo medium, however, is the simplest, as it does not require serum, while the testicle media must contain a certain amount of homologous serum to insure multiplication of the virus. In rabbit kidney tissue, a medium suitable for the cultivation of vaccinia virus, pseudorabies could not be grown.

The pathogenic properties of pseudorabies virus were not altered during the 49 serial culture passages and there were merely quantitative differences between the cultivated virus and rabbit brain passage virus. This was to be expected from the experience of others, since, so far as we know, no filtrable virus has changed its pathogenic properties qualitatively during the course of its cultivation *in vitro*.

SUMMARY

Pseudorabies virus has been cultivated in series in rabbit testicle, guinea pig testicle, and chick embryo media, and its growth requirements have been studied. Intranuclear inclusions, similar to those produced by pseudorabies virus *in vivo*, have been found in rabbit testicle cultures. The virus has not changed its pathogenic properties for rabbits, guinea pigs, or mice during the course of cultivation.

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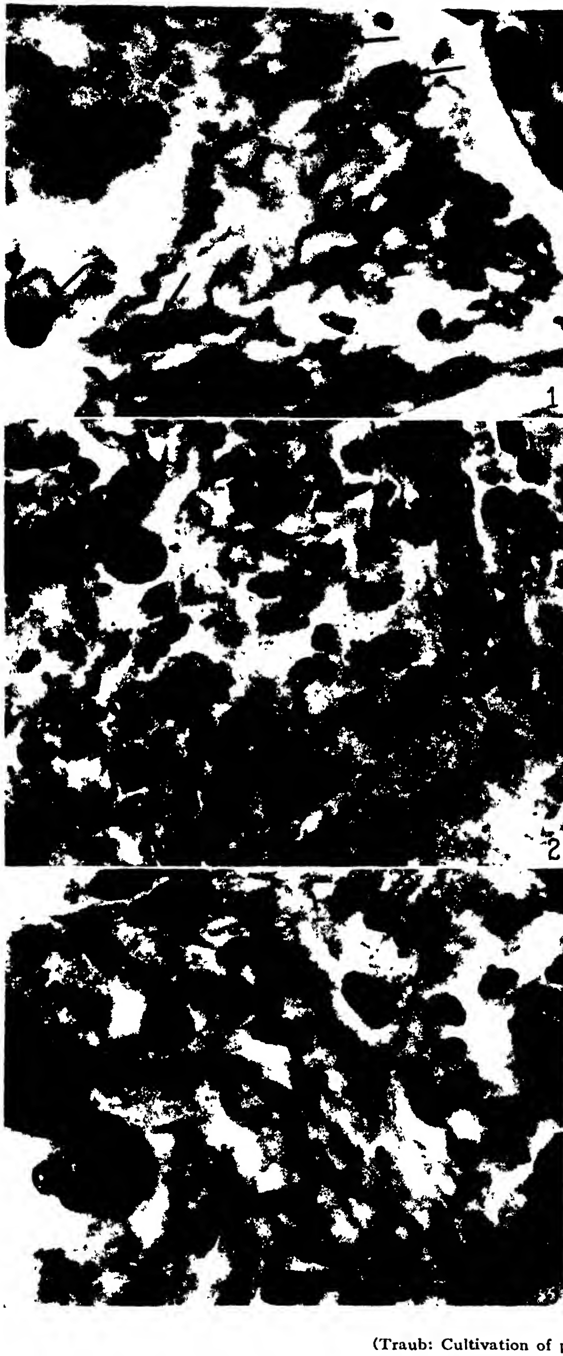
EXPLANATION OF PLATE 40

FIG. 1. Section through a piece of rabbit testicle tissue from a culture incubated for 2 days. Intranuclear inclusions appear in interstitial cells. Hematoxylin-eosin. ×1,033.

FIG. 2. Section through a piece of rabbit testicle tissue from a culture incubated for 4 days. General necrosis. Chromatin has disappeared from the nuclei in the seminiferous tubule (right side of picture). Nuclei of interstitial cells karryorrhectic. Inclusions no longer distinct. Hematoxylin-eosin. ×804.

FIG. 3. Section through a piece of rabbit testicle tissue from uninoculated control media incubated for 4 days. Necrosis much less advanced than in Fig. 2. Nuclei of seminiferous tubule (right side of picture) still contain chromatin. Interstitial cells fairly healthy. Nucleoli still visible. Hematoxylin-eosin. ×804.



(Traub: Cultivation of pseudorabies virus)

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FOCAL CELL REACTIONS IN TUBERCULOSIS AND ALLIED DISEASES

BEING THE WILLIAM SIDNEY THAYER AND SUSAN READ THAYER LECTURES FOR 1933

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PART I. THE EPITHELIOID CELL REACTION

In a problem as complicated as the relation between two living organisms intimately associated as host and parasite—a problem repeated with variations in every infectious disease—the mode of approach determines more or less the information obtained, and with many factors remaining unknown the results of inquiry may differ radically. The experimental method of analysis conveniently pursued in modern laboratories has dominated the field of operations. The final goal, however, is an understanding of the manifestations of the disease as a whole, that is, as it occurs spontaneously. To bridge the gap, the experiment must approximate the natural disease ever more closely, but the experiment itself becomes thereby more complicated as the model approaches the natural condition to be imitated.

Tuberculosis has been exploited chiefly by the use of cultures and culture products of the tubercle bacillus on animals, more particularly by observing the results of inoculations and injections upon normal and partly immunized animals. Latterly, it has been aided directly and indirectly by tissue cultures. Thus far the comparative method has not had its proper share in the analytic problem. The bovine disease has been all but neglected. The spontaneous disease in monkeys has not received the attention it deserves. In the animal forms of this disease, all stages are accessible to study. Though the comparative study of spontaneous tuberculosis has its weak points and defects, the experimental study is not free from them. It suffers from several difficulties which become conspicuous when we endeavor to transfer results to the spontaneous disease. In all cases alien hosts

are used, the bacilli are more or less saprophytized, the dose administered is enormous compared with the single bacillus starting the natural focus. The natural channels are circumvented by direct inoculation and the general manifestations of a diseased state are highly exaggerated and distorted. The habit of accelerated multiplication of the saprophytized or culture bacilli acquired on artificial media is carried over into the inoculated animal. Both in this regard and with reference to the metabolites and the somatic substances, the experimental disease is influenced by a fairly extensive shift of these substances as compared with the spontaneous disease, and this changed concentration cannot but have a distinct influence on the type of cells stimulated and the course of the local reaction. The speeding culture bacillus becomes more vulnerable with increasing growth acceleration. The quantity and quality of the tissue reaction change. Phagocytosis becomes modified in extent or in the character of the participating cell groups.

The most outstanding work illustrating the effects of the different constituents of the tubercle bacillus as isolated and determined by Anderson has been recently published by Sabin and her associates (1). Briefly stated, "the lipoid portion of the organisms, notably a phosphatide constituent, possesses the capacity of producing tubercular tissue, *i.e.*, epithelioid cells and giant cells. The so-called waxes cause a proliferation of fibroblasts. The acetone soluble fat induces proliferation of all connective-tissue cells and of blood vessels, and causes hemorrhage. The polysaccharide is chemotactic for and toxic to leucocytes. The protein is responsible for fever and in addition causes a proliferation of plasma cells." The task of correlating the relative part played by these building stones of tubercle bacilli in the spontaneous disease starting with a single, living bacillus still remains to be done. In what follows I have adhered as far as possible to the spontaneous diseases and to a considerable degree inferences drawn from the material accessible differ from those on record. The literature of tuberculosis is enormous and it is quite possible, and indeed assumed, that concepts and ideas to be presented have been repeatedly published by others.

The dominant activity of infectious agents in the acute diseases is first local and is followed by invasion of the blood and secondary focal

lesions, if the host survives. The local activity rises with the chronicity of the disease and the focal lesion becomes the important center of activity for both host and parasite. The invasion of the blood is in abeyance until the host begins to fail. With the development of the primary focal lesion two factors appear; first, the active participation of the local tissue cells and some general influence emanating from the primary focus which modifies certain cell groups in the entire system and produces both an increased resistance of these cell groups to subsequent invasion as well as a heightened sensitiveness designated as allergy. This general systemic influence gives rise to cell disturbances often of diagnostic value in the living animal. In clinical medicine the rise and decline of mobile cell groups in the circulating blood has been focused upon because it is the readily accessible part. It represents a disturbance to be explained by some focal process, usually a concentration of parasites or bacteria and certain host cells. What goes on in these concentration camps is not the whole story of the host-parasite conflict, but it represents the major share of that conflict. The leucocytic formula of the blood is a mere index of it.

In animal diseases the focal gatherings of such cells are more easily approached, because animals may be killed at any stage. The focal gathering of polymorphs leading to abscess formation is the most frequently observed occurrence. The remarkable chemotactic affinity of eosinophile cells for animal parasites is always a striking phenomenon. Myriads of these cells, often migrating *en masse* through intact epithelial layers, gather in the presence or in the wake of some living parasite and form veritable eosinophile pus. They do not appear to undergo the disintegrative changes observed in the focal aggregations of the neutrophile cells. The regular appearance of lymphocyte gatherings in certain pathological and post-pathological states is also a familiar occurrence. It is pertinent to inquire whether the chemotactic response of these cells is due to more than one substance, or whether there is some one common ingredient or component in the various stimulating agents that governs the gathering of these three cell groups and accounts for their special movements.

In the endeavor to formulate in a general way the cell movements in the presence of infectious and parasitic agents, I have conceived the activities of both host and parasite as offensive or aggressive and

defensive or protective, without assuming any purposeful behavior on the part of either. These two groups of activities, developed through trial and error methods of nature, range themselves under humoral and under immediate cellular activities on the part of the host, and under metabolic, diffusible, and under cell-bound or somatic substances on the part of the parasite. Those substánces of the parasite secreted and discharged during life and finding their way throughout the host organism must be met by humoral substances, either naturally on hand or produced under stress by cell groups as are antitoxins. Those bound to the parasite would be met by contact with or ingestion by host cells. This grouping or classification of reciprocal, interacting forces agrees well with the antiblastic theory first presented by Ascoli (2) and developed experimentally by Dochez and Avery (3). The humoral factors inhibit certain vital functions of the parasite or neutralize their injurious action, and certain host cells then ingest and intern the parasite, either gradually disintegrating it or allowing it to die of inanition.

Aschoff (4), in a discussion of inflammation, distinguishes between a general and a local defensive reaction. The general reaction includes fever, leucocytosis, and antibody production; the local reactive processes involve parenchyma, vascular system, and supporting tissue framework. Applying this theory to the foci of cell reactions as developed in certain diseases, we may further postulate that cell aggregations forming abscesses are in a sense equivalent to phagocytosis by single cells. In the abscess it is the interaction of large numbers of one or several types of cells with or without phagocytosis which not only protects the rest of the host system from invasion, but which also protects, in a sense, the parasite, for here the bacteria may be found in large, often immense numbers, whereas the rest of the system is sterile. Any breaking away of the abscess contents internally would continue the disease. The abscess either discharges its contents outwards, or the contained bacteria gradually decline in numbers and eventually die out.

All studies of focal cell reactions have revolved around tuberculosis, that classical example of a focalized disease. Yet the immense amount of patient research centering in the tubercle has not brought unanimity in the interpretation of its various phases. The variables

involved presenting a different aspect with every approach lead to diverse views. If we study experimental tuberculosis in guinea-pigs, our views will differ from those of others who study it in rabbits. Human tuberculosis differs slightly from bovine, and both distinctly from avian tuberculosis. We arrive at varying results according to the cultures we use, the age of the strains, the period allowed for the span of the experimental disease. Finally, experimental tuberculosis is definitely governed by the number of bacilli introduced.

It has been my good fortune to have had under observation for a number of years two infectious bovine diseases which have many characters in common and many divergent. Bovine tuberculosis and paratuberculosis are sufficiently alike to have induced the first students of paratuberculosis to describe it as tuberculosis. Only when the bacillus could not be cultured on media on which bovine tubercle bacilli will multiply, and when laboratory animals failed to respond to inoculation, was the non-identity recognized. A comparative study of the lesions of the two bovine diseases, supplemented by the isolation and culture of the two types of acid-fast bacilli, brought to the surface a few suggestive facts which I am using as a basis for certain inferences. Several other diseases have been drawn into the comparative study, and the result is a kind of rounding up of miscellaneous observations extending over years.

Although bovine tuberculosis has played such a conspicuous rôle in the operations of public health agencies as well as in the economics of agriculture, and the bovine bacillus such a prominent part in experimental studies designed to throw more light on the human disease, comparatively little has been written on the main, obtruding characters of the spontaneous disease. The relatively short life of cattle eliminates to a large degree long-standing superinfected or reinfected types. A number of years ago (5) I pointed out that if all individuals of a herd of cattle infected with tuberculosis are killed at any one time, a large number—in some herds towards 90 per cent—is in the early lymph-node stage. The respiratory form predominates over the intestinal form, although pastured animals may have tuberculosis of the retropharyngeal and mesenteric lymph nodes which suggests alimentary infection. In the respiratory form the dorsal, mediastinal and bronchial nodes are involved. The lungs themselves

are in many cases entirely free. When foci occur, they are not limited to the apical lobes but may be found in any lobe, chiefly, however, in the most expansive diaphragmatic or lower lobes, clearly indicating direct aerial infection. Foci in different stages and therefore evidence of infection at different times are absent. Rarely, caseous pneumonia follows the aspiration of discharging primary foci into other regions of the lungs. In the dorsal mediastinal nodes, the primary infection is frequently manifest in small tubercles, 1 to 2 mm. in diameter, situated in the zone in which carbon and dust particles are deposited. These I assume are due to a single bacillus or a compact mass of a few bacilli carried thither from an alveolus by way of the lymphatics. Unfortunately, the earliest pre-necrotic stage I have not seen. To the naked eye the small tubercles in this stage simulate the normal tissue closely and are overlooked. This primary tubercle, when first detected, consists of the irregularly jagged necrotic center, an enveloping zone of epithelioid cells, gradually giving way to fibroblasts, and an outer distinct capsule of these cells. Giant cells are usually absent from the primary tubercle. Similarly, tubercle bacilli are scarce and frequently searched for in vain.

Many researches have been focused on this primary seat of tubercle bacilli, but since it comprises the life of two quite different organisms, the host cell and the tubercle bacillus in intimate contact, two quite different types of cell metabolism and the eventual, rapid or slow destruction of both cell and bacilli, the final interpretation of the building up and the involution of the tubercle must await much more penetrating cell studies than are available at present. All we can do is to encircle the problem a little more closely and propose suggestions which may or may not point in the effective direction.

Leaving for the moment this disease and turning to the second bovine disease due to acid-fast bacilli indistinguishable morphologically from tubercle bacilli, we are confronted with certain suggestive likenesses and differences (6). The activities of the paratubercle bacillus are limited to the mucous membrane of the intestines, extending in some animals to the submucosa and always to the mesenteric lymph nodes. The disease starts in the lower ileum and spreads in both directions, reaching both the duodenum and the rectum in certain active types of the disease. The degree of infiltration and swelling of the mucosa

depends on factors of resistance and virulence. Disease may be slight and not recognizable with the naked eye, or the swelling may be extreme. The mucosa is then thrown into large thick longitudinal and transverse folds. There is no evidence of loss of epithelium or ulceration. Clinically, the disease manifests itself in the form of intermittent or persistent watery discharges and progressive emaciation. There is no definite thermal elevation. Among the regularly appearing reactions found at autopsy is an extensive phagocytosis of red cells in the spleen and gradual conversion of the inclusions into pigment. Evidently the red cells are damaged while circulating in the capillaries of the intestinal villi. More rarely, extensive necrosis of the fat deposits in the abdomen is seen and this is probably due to the infiltration of the mucosa around the papilla of the pancreatic duct, resulting in partial obstruction of the latter.

The histological picture is fundamentally the same in all cases examined but it varies in degree and intensity. In the rapidly advancing type of disease, the entire intertubular tissue of the mucosa consists of cells of epithelioid type. A zone of similar cells, broad or narrow, may be found in the submucosa. In the stationary, resisting type a small focus of epithelioid and giant cells occupies the distal or free portion of all or of only a certain percentage of the villi. When extending to the large intestine the process involves the same tissues. The intestinal tubules, with exceptions to be given, are intact. The mass of epithelioid cells has crowded out all lymphocytes and eosinophile cells normal to this tissue. Applying the carbolfuchsin stain and decolorizing with acids, or acids and alcohol, we find that every cell having epithelioid characters contains acid-fast rods of quite uniform size and slightly shorter than tubercle bacilli. This difference in size largely vanishes in cultures, however. The number of bacilli within cells may be so great that the cell itself appears as a dense red mass. Since this occurs in animals killed and promptly autopsied, it is not the result of post-mortem multiplication. In the restricted disease with small foci in the villi, bacilli are always present in the cells but are few in number. Although the epithelioid cells may be loaded with bacilli, they appear normal when stains, such as hematoxylin, are used which do not bring out the contained obscuring bacilli. In eosin-methylene blue sections the bacilli may be detected as ex-

cessively minute bluish points in the cytoplasm. As stated above, the mesenteric nodes are always invaded. Epithelioid cells filled with bacilli occupy the follicles and cords and tend to obliterate the peripheral lymph channels through expansion and pressure. The size of the nodes is not, however, appreciably increased.

Among the differences noted in the cell reactions manifested in the presence of tubercle and paratubercle bacilli, the irregular, indefinite extension of the epithelioid tissue in paratuberculosis, as contrasted with the focal, definitely limited spherical structure of the tubercle, is noteworthy. A second differential character is the total absence of necrosis or caseation even in the most extensive infiltration of paratuberculosis. Associated with these characters is the absence of any tendency to fibrosis or to bounding or limiting the epithelioid areas by capsule formation. The respective seats of the local disease in the two diseases differ strikingly. The failure of the paratubercle bacilli to multiply beyond the mesenteric lymph nodes is not explainable at present. The favorable nidus in these nodes should be present in lymph nodes elsewhere, unless some special product of digestion is needed which might not be available beyond the mesenteric nodes themselves.

The differences in these two diseases cannot at present be correlated with what is known of the bacilli. The paratubercle bacilli do not produce a progressive disease in laboratory animals which are susceptible to the tubercle bacillus. Slight local lesions may be produced and some of the injected bacilli recovered in cultures months later. The culture of the paratubercle bacilli failed on media which favored multiplication of the tubercle bacillus until Twort and Ingram (7) pointed out that the addition of dead tubercle bacilli, or extracts, made multiplication possible on egg media and on glycerine agar. Following Twort's procedure and using a suspension of tubercle bacilli boiled in 50 per cent glycerine as an addition to egg media, I have experienced no difficulty in obtaining pure cultures from every animal autopsied.

Naturally, the problem of the origin, source, and nature of the epithelioid cells which form the matrix in which both types of bacilli are found could not be thrust aside, for it has agitated the minds of pathologists since Virchow's day. Baumgarten (8), Orth, Ziegler,

Thoma, and other pathologists regarded the epithelioid cells as derived from the fixed cells of the connective tissue. Metschnikoff regarded the tubercle as a mass of immigrated macrophages. Since the formulation by Aschoff of the reticulo-endothelial system as a collective concept of variously functioning cells of the lymphatic and connective tissue structures, the source of epithelioid cells has been conveniently referred to it. Some writers have been more definite in regarding vascular and lymph epithelium as precursors.

The variations in the cellular reactions of the mucosa from animal to animal affected with paratuberculosis gave rise to certain suggestions as to the development of the epithelioid cell which favored the views of the older pathologists that it arises from cells *in situ*. Most sections present the picture of large areas of epithelioid cells, stuffed with bacilli. The cells may be still with a single nucleus, or they may have two or even more. In certain cases the adenoid reticulum consisting of still fusiform elements also contains bacilli, either incorporated with or attached to the cytoplasm. Similarly, when the still normal membrane near the muscularis mucosæ or the submucosa itself is scrutinized, minute clumps of bacilli may be detected attached to still fusiform elements. These latter may be either alone or already associated with epithelioid-like, bacilli-containing cells. Without drawings it is difficult to convey the appearances. Photographs do not present the details clearly. On the basis of these observations, the theory has gradually taken shape that the epithelioid and its derivative, the giant cell, are produced by contact with the bacilli which stimulate in some way the growth of the cell cytoplasm and eventually become enveloped by it. The epithelioid cell is conceived as a hypertrophic pathological product of a normal cell. It may be rapidly destroyed as in tuberculosis. It may be stimulated in various degrees by the parasitic inclusions or contacts. It is a sick cell with heightened capacity to digest the contents, or soon overcome by the toxic character of the inclusion. The host-parasite cell is thus an unstable product with many degrees of susceptibility and resistance from early necrosis to complete resistance and final indifference to the presence of the bacilli. Each host-parasite combination defines the character of the resulting lesion, its morphology and growth limitations.

If we contrast the cell behavior in tuberculosis and paratuberculo-

sis, certain interpretations appear justified. The spherical, limited structure of the tuberculous lesion indicates some central radiating activity missing in the paratubercle bacilli. The prompt central necrosis may be due either to a high initial fragility of the normal tissue cells in the presence of a toxin, or to a rapid swelling or hypertrophy of the cell cytoplasm checking normal nutrition, or both factors may be at work. The declining concentration of the toxin outward leads to the epithelioid cell mantle and lastly to the fibrous capsule. A high virulence favoring rapid multiplication would lead to dissemination of the tubercle bacilli, before a shutting in of the primary focus, through the system. This occurs in experiments with small animals. The inference is warranted that there is a certain definite relation between the tubercle bacilli and the natural host similar to that of other host-parasite combinations. This relationship manifests itself in the localization and multiplication of the bacilli in certain tissues, as well as in the modification of the tissue cells themselves in the presence of tubercle bacilli. Degrees of immunity and susceptibility disturb this relationship towards greater multiplication of the bacilli or their suppression and towards a changing behavior of the tissue cells. The relation resembles the adjustment of a chemical reaction to a certain equilibrium shifted one way or the other by changes in the concentration of the constituent substances.

In paratuberculosis the evidence points to the absence of any diffusible toxins. The disease progresses by contact of bacilli, which have escaped from infected cells, with fresh tissue cells. In tuberculosis the bacilli appear to influence cells through metabolic products. Epithelioid cells are probably developed without direct contact with bacilli. The general scarcity of bacilli and their absence from most epithelioid cells, unless current staining methods are at fault, warrant this inference. Necrosis or caseation may be the direct result of toxins. When the tubercle bacillus is of a lower order of virulence but of greater persistence, necrosis may be due to the gradual expansion of the epithelioid syncytium which enters a slow nutritional death through gradual blocking of the vascular supply.

It is highly probable that the epithelioid cell has been traced to too narrow an ancestry and that different views among pathologists may in part be ascribed to this conception. It is conceivable that, besides

physiological differences among bacteria, special types of virulence among the same bacteria may bring different cell types into the epithelioid class. A swelling of the cytoplasm and an enlargement or edema of the nucleus with changed condition of the chromatin may approximate different cells to a superficially identical type. In rabbits the pulmonary lesions following injection of bovine tubercle bacilli have in some foci presented to the writer the appearance of alveolar cells becoming epithelioid by an increase or hypertrophy of the cytoplasm.

The course of the two bovine diseases differs, in that paratuberculosis appears to be continuous in its progress, while tuberculosis is saltatory. The tubercle develops and becomes encapsulated, and unless bacilli escape from this primary focus the disease quiets down. When escape does occur, there is a sudden revival of the symptoms with the appearance of a second crop of tubercles. Occasionally, the shock of this secondary attack kills experimental animals, such as rabbits and guinea-pigs, over night. There are however differences between the first and the second stage. The secondary tubercles remain small. Necrosis is less extensive or entirely absent. Giant cells are regularly present. This change in reaction is associated with a changed attitude of the tissues associated with allergy. The well-known tuberculin reaction is a product of this changed state. It is obvious, in view of the appearance of allergy, that besides the local initiating tuberculous process there is a humoral factor issuing from this local focus which influences the entire system and leads to a different behavior of the reticulo-endothelial system and the escaped bacilli towards one another. This partial immunity tends to be ignored because it is partial. Upon it depends any assistance that may be expected from any form of vaccination or serum therapy. Allergy could not develop without the wide diffusion of some diffusible antigenic product of the tubercle bacillus. The more highly parasitic or specialized paratubercle bacillus does not stimulate any allergic condition, and the process does not tend to become self-limited unless genetically predetermined. There is no paratuberculin reaction of sufficient significance to be of any value in diagnosis. The metabolism of the bacillus is of a character which is spent in contact with and within the epithelioid cell, and systemic effects are negligible. Even with extensive disease the temperature is about normal.

The changed tissue reaction associated with and following the primary focus in tuberculosis involves a partial immunity of the entire reticulo-endothelial system. Certain diffusible substances set free from the primary focus evidently affect certain tissue cells every where to become more resistant to certain products of the tubercle bacillus. The necrosis is restricted or prevented. The bacilli from a primary focus, now controlled by certain antibodies, are limited in their activity to the formation of giant cells in which they are lodged like neutral foreign bodies. With the partial or complete neutralization of its offensive or aggressive activities the tubercle bacillus may still have defensive or protective activities within the epithelioid cells which keep it alive for variable periods of time.

In looking about for pathological tissue activities to test the conception of the epithelioid cell as presented, certain cell activities in the bovine intestinal tract aroused my attention. In inflammations of the small intestine the swelling of the distal portions of the villi as a result of lymphocyte infiltration tends to compress the mouths of the tubules. The lumina expand and may become filled with mucus. This peculiar lesion was first observed in the study of scours or early diarrhea in calves. Although not entirely absent in very young animals, the distended tubules were seen in largest numbers in calves from one to two months old and in intestines not macroscopically abnormal. It was at first thought that the distended tubules were open, but serial sections did not confirm this assumption. A certain number of calves go through an early period of intestinal disturbance, due chiefly to multiplication of *B. coli* in the lower small intestine, which may account for the interstitial infiltration observed. The occluded tubules vary more or less in shape, condition of lining epithelium, and contents. The varying size of the lumen is probably due to the quantity of mucus secreted into the tubule, for a few tubules contain only mucus. All the rest are partly or wholly filled with cells which have migrated into them. It is probable that the mucus forms a kind of supporting medium for the invading cells. Most frequently the invading cells are of uniform type, with only a few of other types present. The commonest cell form present is the eosinophile leucocyte which is abundant in the interstitial tissue of the normal bovine intestine. These cells form dense molds filling the lumen, and are in

various stages of disintegration. The second type of cell, often mixed with eosinophiles and in some instances the major type with but few other cells present, is the cell known as macrophage or clasmatocyte. In their expanded state in the empty tubule these cells are nearly circular in optical section and measure about 25 microns in diameter. In sections of fixed tissue the cytoplasm is uniformly bubbly and appears as a meshwork with occasional inclusions of masses of chromatin, probably from ingested leucocytes. It is bounded by a narrow, slightly denser outer zone (ectoplasm). The nucleus is oval with chromatin distributed in small masses on the inner surface of the delicate nuclear membrane. Rare mitoses indicate division after entering the tubule.

The epithelium of the clogged tubules is rarely intact. Most frequently it is flattened and thinned out by compression, particularly in the part nearest the intestinal lumen. It would require much more abundant material with detailed history from birth, as well as certain prenatal data, to formulate an explanation of the preponderance of one or the other migratory cell type in the occluded tubules. Possibly some form of microbic life may have been imprisoned in the tubule to account for the cell type present. In one group of calves the cells maintained the more or less discrete form of the mobile clasmatocyte, but in another they tended towards the epithelioid cell type and giant cell formation. They formed irregular syncytia in which cell outlines were no longer distinct.

Among the changes in the mucosa in paratuberculosis a similar occlusion of tubules presents certain interesting problems. The swelling of the distal portions of the villi where the epithelioid cells first appear naturally tends to compress the mouths of the tubules, some of which become greatly distended. In those cases which are characterized by an active proliferation of epithelioid cells in the intertubular tissue, and in which the distended tubules are embedded within the epithelioid areas, the tubules are the scene of an ingrowth of epithelioid cells. The ingrowth is attached to the basement membrane where the epithelium has disappeared. The ingrowing cells form a coherent mass with an occasional mitotic figure in it. It is not clear whether this mass grows by cell division at the root of the mass and is pushed inward or within the mass and on its periph-

ery. Mitotic figures have been found near the free tip of the mass. That the proliferation proceeds from fixed cells in the subepithelial zone seems well established by the fact that all ingrowths are attached by a pedicle to the subepithelial tissue bared of its covering of epithelial cells and that in some instances there is a cell ingrowth from the greater part of the circumference of the tubule. In paratuberculosis all the ingrowing cells contain bacilli, either few or many, and it is probable that the bacilli are passed on in the division of the cytoplasm to the daughter cells. The entire ingrowth or plug is thus made up of epithelioid cells containing bacilli. There is some evidence that the epithelioid-cell mass erodes the epithelial layer and then pushes its way into the lumen. On the other hand, the free cell, or clasmatocyte, has not been found in relation to any break in the epithelial lining and its mode of entry into the lumen is not known. It was present only in tubules still embedded in normal intertubular tissue. The passage of polynuclear, amphophile and eosinophile cells and of lymphocytes, into the lumen of passages lined with epithelium is effected by a kind of digestive process. The epithelium becomes filled with vacuoles containing these migrating cells. The method used by the larger clasmatocytes has not been seen.

The plugged and expanded tubule presents a kind of tissue culture *in vivo*. There is little doubt that the large cells appearing in the lumen in a relatively normal submucosa, or in a relatively normal environment in a diseased mucous membrane, are clasmatocytes and different from the ingrowing cell mass in a paratuberculous environment. It is difficult to avoid the inference that the paratubercle bacilli are the immediate stimuli to the cell ingrowth. Each cell contains them. It is also difficult not to consider them as derivatives of fixed cells modified by the bacilli within them. A third inference seems warranted, that they are capable of corroding and destroying the superposed epithelial cells. That this corrosion is not going on in many other areas may be due to the fact that the epithelial cells of the plugged tubules are no longer normal.

The total absence of mitotic figures in the main seat of the disease between the tubules and their occasional presence in the ingrowth tempt one to conclude that multiplication is inhibited in the adenoid

tissue by surrounding pressure and that all epithelioid cells there are derivatives of existing reticulo-endothelial cells.[1]

The giant cell as an integral part of the tubercle has held the attention of pathologists, even before the discovery of the tubercle bacillus, when it served as an important diagnostic character. Koch referred to it repeatedly in his monograph. He found it regularly present in the center of the tubercle. Bacilli were always present, even up to fifty or more in some cells. He observed the destruction of giant cells by multiplying bacilli. Today the derivation of giant cells from epithelioid cells or their progenitors is generally accepted. Their presence, however, is not a regular occurrence in tuberculous lesions. In fact, a study of this disease, either spontaneous or induced in different animal species, clearly points to this cell as an indication of natural or acquired resistance, since it is rare or absent in most experimental animals and in the primary bovine foci. In bovine tissues it is regularly present in secondary lesions in the neighborhood of primary foci. The lesion associated with the giant cell is small, occasionally limited to this cell and a mantle of lymphocytes. Necrosis is slight or absent. The cell may be regarded as the result of reduced stimulation by the infectious agent. We have seen that in both tuberculosis and paratuberculosis the epithelioid cell tends to grow into empty spaces by mitotic multiplication. This tendency reduced in intensity would lead to multiplication of nuclei only and a giant cell as the ultimate result. In our examination of tissues the giant cell has contained but few paratubercle bacilli and rarely any in tuberculosis. It is probable that Koch's material was not from an average case of the bovine disease. The giant cell resulting from reduced host-parasite activities may be conceived as the product of a changed disposition of the reticulo-endothelial cell complex. This latter state may be the result of natural resistance or acquired through the dissemination of products from the primary lesion. Stimulation

[1] The tendency to ingrowths in bovine tissues under pathological influence is seen under various conditions. In tuberculosis, ingrowths into the pleural cavity may form very large pendulous masses. Ingrowths from tuberculous peribronchial foci into the bronchi are present in pulmonary tuberculosis. Ingrowths following pneumonia were recently described by the writer (24).

to multiplication of nuclei may occur even in epithelial cells. The writer (9) described intestinal epithelium undergoing multiple nuclear division in the presence of coccidia in the cytoplasm.

The changed reaction of the host tissues to a secondary crop of tubercle bacilli discharged from a primary focus is intimately associated with tuberculin sensitiveness. It is well known that Koch in his early experiments called attention to this changed reaction in the local lesion produced by a second injection of tubercle bacilli. The reaction points to a fixation of the injected bacilli locally, due to an acceleration of the tissue response as compared with the primary reaction. The question whether this allergic condition spreads out beyond the specific infectious agent is of more than ordinary significance. P. A. Lewis and Dorothy Loomis (10) observed that tuberculous guinea-pigs develop much more anti-sheep amboceptor than do controls when given like amounts of sheep red blood corpuscles. Certain heterologous reactions have been observed by the writer in bovine tuberculosis. The carbon pigment coming from the lungs and deposited in the mediastinal and bronchial lymph nodes does not give rise to any unusual cell reaction ordinarily, but in certain nodes having small foci of tuberculosis most of the cells containing the pigment become giant cells. In 1910, the writer (11) published two observations which may be interpreted as a more general allergic condition towards alien material. In a cow inoculated into the thorax with a human type of tubercle bacilli, there was found at autopsy, two months later, on the omentum a flattish, convex, sessile mass of tissue with a circular base about 2 cm. in diameter. The tumor-like mass was pinkish gray. When sections were prepared of the fixed and hardened mass at right angles to the base, it was shown divided into contiguous circular areas, about 3 mm. in diameter, by fibrous tissue. There were about twelve such areas forming the median section. In the center of each was a foreign body readily identified as a nematode in cross-section. Immediately around the body-wall of every cross-section of the worm was a narrow necrotic zone, together with an occasional foreign-body giant cell and some fragments of the worm. The rest of the enveloping zone was made up of lymphocytes. The worm was identified as the common filaria of cattle, normally coiled up in the omentum at a certain stage. The oviducts and uteri

were filled with ova and embryos. In one lung of this animal another roundworm, not identified, had become imprisoned in a mantle of lymphocytes. In another animal which had received several intravenous injections of tubercle bacilli, a similar, smaller worm-tumor was found on the omentum with a filaria buried in it. It might be argued that tubercle bacilli had become lodged near the worm and had given rise to the lymphocyte focus. But, in the first cow, the injection had been made into the pleural cavity where no lesions developed and the characteristic arrangement of cells around every coil of the filaria could hardly be expected if tubercle bacilli had formed the immediate stimuli. The cell mantle was in fact oriented around the body of the worm. This aggressive attitude towards carbon pigment, on the one hand, and animal parasites, on the other, not manifesting itself in normal animals, is indicative of some modified generalized activity on the part of the mobilizable cell groups. This activity may also be accentuated by an especial individual allergic sensitiveness peculiar to certain individuals.

PART II. THE SUCCESSION OF CELL REACTIONS

The variously complex or multiple antigenic structure of parasites necessarily involves cell reactions of different types following one another in the course of any reactive or hostile host-parasite association. Hence we should expect tissue changes in the development and decline of such an association to follow certain definite courses, complicated however by variations in both qualitative and quantitative directions which might obscure the fundamental reactions. In bovine as in human tuberculosis, the tubercle is frequently associated with lymphocytes forming mantles around stationary or subsiding activities of the tubercle bacilli. In paratuberculosis the mucosa is infiltrated with lymphocytes when the primary epithelioid proliferation is slight. In neither bovine disease have polynuclear cell invasions been seen in any stage. The relation of this cell to tubercle formation has been the theme of many students of the tissue reaction in tuberculosis and has recently been discussed by Opie (12). Polymorphs have been associated with the earliest stages of the experimental disease by many observers. Recently Vorwald (13) has emphasized this position following certain experiments. In the

experimental tuberculosis of guinea-pigs following the injection of tissue products containing bovine bacilli or of cultures of freshly isolated bovine strains, the central invasion of tuberculous foci by polymorphs, forming an abscess, was not infrequently noted in animals tending towards an increase in weight. It was thought at first that this late process was associated with definite bovine strains, but this assumption could not be substantiated, since the infiltration could not be reproduced regularly. The late invasion of a tubercle by polymorphs suggests either beginning gradual necrosis of tissue or death of contained bacilli giving rise to special chemotactic substances. The relation of polymorphs to the beginnings of tubercle formation is another question. It is doubtful if it ever occurs in the spontaneous disease. The injection of highly saprophytized bacilli in large numbers into the circulation is a very different procedure from the deposit of a single bacillus by way of the lymphatics into a lymph node.[2]

As a contribution to the general theme, avian tuberculosis was drawn into the field of work. In the spontaneous disease tubercle bacilli are usually very numerous in the central necrotic masses of the focal lesions and in the enveloping secondary tubercles. The necrotic mass becomes a foreign body in the sense that a circle of epithelioid and giant cells encloses it. To modify the natural process, freshly isolated bovine bacilli were injected into a wing vein so as to cause a general distribution of the bacilli. Though the bovine type makes no headway in poultry and the injected animals remain well, certain cell activities in liver, spleen and lungs, indicate the close relationship of the races of bovine and avian types. Minute collections of cells appear in the organs mentioned. They may be entirely lymphoid, or entirely epithelioid, or a mixture of the two cell forms. When the lymphocyte foci appeared first and if the bacilli possessed more than

[2] The writer would make a distinction between tuberculous foci developing in tissues shut out from contact with the exterior, such as lymph nodes, and those developing secondarily in the respiratory, renal, digestive, and genital tracts. In the disease of guinea-pigs due to inoculation with the bovine abortus bacillus, lesions in the epididymis were always associated with suppurative processes, whereas this did not occur in spleen and lymph nodes. Possibly some local filtrable virus may inhabit these exposed tracts and associate itself with the bacteria.

the usual resistance and began to multiply temporarily, epithelioid cells containing bacilli appeared in the center of the small lymphocyte group. If the bacilli did not multiply, no further change took place. Later on, the epithelioid foci appearing with or without preceding lymphocyte gathering were again invaded by lymphocytes as an indication that the active process was over. The appearance of epithelioid centers in the lymphocyte foci is attributed to the swelling or hypertrophy of the cytoplasm of the reticulo-endothelial elements in the focus under the influence of an antigen of the bovine bacilli multiplying abortively and different from that causing the primary lymphocyte gathering.[3]

Some time ago it was pointed out by the writer (14) that whereas the epithelioid foci following injection of the bovine race of *Brucella* into guinea-pigs were not invaded by polynuclear leucocytes, this was quite regularly seen in epithelioid foci due to the porcine race. Such foci might become large abscesses. Here the assigned reasons for the difference were either a rapid proliferation of epithelioids into large foci and hence of less robust character which became necrotic centrally, or the destruction of the enclosed bacilli which gave rise to chemotactic substances. These illustrations bring out the primary phase as that of epithelioid cell proliferation, upon which is grafted the subsequent polymorph invasion. Inasmuch as polymorphs appear sooner or later in most tissue injuries and respond to a variety of chemotactic stimuli, their behavior does not define the condition which preceded their immigration. All that can be postulated is the dual process: first, epithelioid cell production, and second, polymorph invasion—under certain conditions determined either by degrees of local tissue resistance or by bacterial lysis.

A gap would be left in the present discussion if macrophages or clasmatocytes were not included. Their rôle in certain acute infectious diseases has been especially emphasized by Gay (15) and associates in recent years. The invasion of macrophages into closed distended intestinal tubules under conditions departing little from the normal has been mentioned above. Their association with suppura-

[3] In a case of spontaneous tuberculosis in a monkey there was little or no necrosis of the epithelioid center and extensive secondary immigration of polynuclear leucocytes.

tive processes has been widely recognized. When a non-fatal dose of *B. coli* is injected into the peritoneal cavity of guinea-pigs the immigration of polymorphs is followed after a few days by macrophages which ingest the former. In several guinea-pigs the exudation had become a pure culture of macrophages. In experiments designed to trace the source of agglutinins in the cow's udder, living *abortus* bacilli were injected into the udder ducts. Within 24 hours a copious immigration of polynuclear cells into the secreted milk took place. As this subsided, macrophages began to appear in the milk. Any association of macrophages with the local formation or disintegration of tubercles or in the expanding proliferation of epithelioid cells in paratuberculosis was not observed at any time. The macrophage is either tardily stimulated by the agency that promptly calls out polymorphs or is influenced by another substance. It may be stimulated by the degeneration of polymorphs themselves. The late appearance may also be due to the sessile character and the apparently limited and localized supply of these cells. In the illustrations given, the macrophage appears to perform the task of cleaning up or scavengering after the battle has subsided in favor of the host. Views on the sources of the mobile and sessile protective cells of the mammalian host may be expected to differ among cytologists owing to the complexity of the subject and the difficulties surrounding interpretations. Sabin, Doan, and Cunningham (16) regard clasmatocytes as derived from spleen endothelium, while M. R. Lewis and W. H. Lewis (17) consider mononuclears, macrophages, and epithelioid cells as merely different phases (functional variations) of the same cell type.

To trace events in the tuberculous lesion from start to encapsulation presupposes an adequate conception of the fate of the tubercle bacillus in it. As yet it is impossible to give a convincing account of what happens to it. In paratuberculosis it is probable that the numerous bacilli survive in the cells and are eventually cast off. In bovine tuberculosis bacilli are always scarce and frequently not found, although inoculations with tuberculous products are regularly followed by disease in guinea-pigs. Three possibilities present themselves. (a) Multiplication and destruction go on simultaneously while certain bacilli are acquiring a resistant envelope and are finally protected in the necrotic tissue. (b) There is at first rapid multiplication

checked later by immune reactions. (c) Multiplication is restricted from the start and all progeny is preserved for discharge into another host. The last requirement corresponds to a well established parasitism. The abundance of the progeny is adjusted to the losses inevitable in reaching another host. Bacterial diseases, however, are not well established host-parasite associations. The disease itself is an index of the want of balance. Only when evolutionary processes have largely eliminated disease by a proper host-parasite equilibrium can we regard the third condition as prevailing. This condition appears to be more or less attained in the bovine disease, for secondary, generalized infection is rare, and animals can carry a large amount of necrosed tissue with few outward signs of physiological disturbance. The disease is purely local in many animals and evidence of unbalance is due more or less to a highly unnatural environment.

In the experimental disease in guinea-pigs we face an unbalance, leading to prompt or very gradually approaching death, and the bacilli are more in evidence. When guinea-pigs receive a fairly large dose of recently isolated bovine bacilli (0.1 mgm.) into the peritoneal cavity they die within three or four weeks, usually quite suddenly and with thorax filled with a serous fluid. The lesions are represented by groups of epithelioid cells undergoing necrosis and contain many tubercle bacilli. If the dose is smaller and the animal survives this critical period, bacilli are very scarce in the lesions. Under these conditions, especially in the acute type, peculiar forms of bacilli are frequently found. One is the drumstick form, in which one end is swollen into coccoid shape. Bacilli may also appear as two coccoid bodies connected by a feebly outlined rod form, or as acid-fast granules or short segments in line. It is tempting to consider these degenerated forms, although proof is wanting. Recently Gróh (18) has described these granules as the progenitors of new bacilli. The destruction of tubercle bacilli introduced experimentally into rabbits has been thoroughly studied by Lurie (19). Taking available facts as a basis we may infer that tubercle bacilli are actually disintegrated in the surviving epithelioid cells. Secondary, metastatic deposits of bacilli are probably confronted by the reticulo-endothelial tissue made more resistant by antigens discharged from primary foci. Humoral

antibodies may also be given off in the original foci and act directly on the bacilli, for wherever the tubercle bacillus lodges secondarily, the lesions resemble one another and the bacilli are either seen in a certain abundance or not at all. This secondary uniformity of behavior of tissue and bacilli can scarcely be due to anything else than humoral activities starting from the primary focus.

In addition to tuberculosis, paratuberculosis, and infectious abortion in guinea-pigs, epithelioid cell proliferation or stimulation may be present, as a precursor to the polymorph invasion, in what are regarded as acute suppurative processes. The disease in which this form of focal cell reaction was observed is the well-known spontaneous streptococcus disease of guinea-pigs which is readily maintained by contacts. This disease appears in the form of abscesses of lymph nodes which may assume egg size before they rupture. Oral infection induces suppuration of the submental and cervical nodes; infection of the limbs, axillary or inguinal buboes. Suppuration of secondary nodes, such as the bronchial and pelvic nodes, when it occurs, inevitably leads to death. The subcutaneous nodes eventually break and the abscesses disappear, often to be followed by others. The streptococcus appears in chains and is hemolytic in type. For comparative studies a more highly virulent race was at hand which developed under the following circumstances. A group of some twenty guinea-pigs from the same population affected with buboes was brought together in 1917 and kept interbreeding in a small enclosure until 1926. The excess of young was removed from time to time. In the latter year the disease appeared to be lagging in so far as fresh infections were rare. It was feared that the infectious agent might be lost and inoculation was resorted to, with the result that all inoculated animals died within a week. Even placing pus or cultures on a shaven area was fatal. The organisms appeared in the form of clumps, no longer in chains, although cultures from this colony made years before grew in chains. The clumping was ascribed to additional capsule formation. It was also thought that another agent had supplanted the original streptococcus in this limited population, but this view could not be substantiated. Recently the unmodified, non-fatal type was discovered in a foreign population of guinea-pigs, and with this and the highly virulent strain aided by partial immunization, the facts to be described were determined.

In the original description of this disease by Boxmeyer (20) reference is made to monocytes in the lymph nodes. In my own examinations of lymph nodes of guinea-pigs in contact with this disease I frequently encountered areas of cells not to be distinguished from epithelioid cells and so closely resembling those of the *abortus* disease in guinea-pigs that for some time I suspected that the lesions were due to *B. abortus* accidentally introduced. The peculiar condition was finally cleared up when the lymph-node abscesses of the spontaneous disease and those of partially immunized guinea-pigs inoculated with the highly virulent strain were examined. The process in brief may be interpreted as follows. The highly virulent type introduced through abrasions produces foci in which the normal tissue becomes promptly necrotic and an immigration of polymorphs takes place without any phagocytosis. Fresh metastatic foci develop and death comes in four to seven days. In guinea-pigs subject to the endemic type or adequately protected through vaccination towards the highly virulent type, the local process may take a different course. There appear at first groups or foci of cells epithelioid in form, even giant cells. These foci become subject to two subsequent processes, either an immigration and infiltration of polymorph cells, or a necrosis followed by immigration to the periphery of such necrotic foci. It is possible to observe in sections of such foci a groundwork of the epithelioid type of cells infiltrated with polymorphs. The current interpretation would be that we are dealing with a nest of macrophages or clasmatocytes which have taken in the polymorph cells. I was under the spell of this interpretation for some time, but I now regard the polymorphs as having invaded the degenerating epithelioid cells. We have then a process much like that in the *B. abortus* disease of guinea-pigs.

This combination of a central nucleus of polymorphs or pus surrounded by a broad zone of epithelioid cells in which scattering giant cells are present has probably been interpreted by pathologists as primary suppuration followed by the formation of an enveloping zone of cells akin to modified fibroblasts. It will be noted that the interpretation advanced is the reverse. It postulates a groundwork of stimulated epithelioid-like cells invaded later by polymorphs. In a search for an explanation of this dual process we may grant that the super-virulence of one strain of streptococci is due either to a maxi-

mum production of capsular substance or to the production of a second, protective substance also capsular but not identical with the first. The normal guinea-pig is unable to cope with this modified coccus, but by immunization, or when this second hypothetical substance is not produced or the original capsular substance appears in smaller quantity, primary necrosis of tissue is replaced by hypertrophy of the reticulo-endothelial groups of cells and the formation of epithelioid, and more rarely, of giant cells, followed finally by immigration of polynuclear cells and softening of the focus. Placing side by side certain selected microscopic foci, about one half the diameter of a 16 mm. field, from the lymph nodes of guinea-pigs inoculated, respectively, with bovine tuberculosis, *Brucella abortus*, and the streptococcus, we should find difficulty in allocating each one to the proper source of infection. Each one represents a background of epithelioid tissue centrally invaded by polymorphs and the whole surrounded by more or less connective tissue in the form of a capsule. Though the morphological basis of the local reaction is alike in the three diseases, there is no reciprocal immunization. The mechanism is the same but the underlying physiological processes are specifically different.

The modification of the cellular types of reaction in accordance with certain differences in the host-parasite relation, such as a difference in the virulence or antigenic concentrations of the infectious agent, may suffice to account for certain earlier controversies in which both parties were in part right. The kind of lesion produced by the bacillus of glanders was the subject of considerable interest as well as controversy some forty years ago. Baumgarten regarded the glanders nodule as intermediate between a tubercle and an abscess. According to him, the nodule is formed by epithelioid cells derived from fixed cells of connective tissue, vessels, or parenchyma. There follow infiltration from the periphery with leucocytes and the resulting softening and disintegration of the tubercle. From the pus obtained from a fatal case of glanders, J. H. Wright (21) produced the experimental disease in guinea-pigs, and basing himself on an examination of the tissues of these animals, he states that the primary effect of *B. mallei* is not a collection of epithelioid cells but necrosis of the tissues followed by invasion of leucocytes. Later Duval and White

(22) came to a different conclusion. They used a strain which had been attenuated by culture and also raised in virulence by a series of passages through guinea-pigs. A study of the lesions in guinea-pigs developed the fact that the virulent passage culture produces exudative changes, whereas the attenuated form is associated with a tubercle-like structure consisting of epithelioid and giant cells. Certain nodules in the lungs of horses were the subject of controversy, Nocard regarding them as due to glanders, while Schütz (23) looked upon them as parasitic in origin. From our standpoint we should regard both as conditionally right. The horse's lung may contain either or both types of infection in which the histological cellular reactions simulate each other.

The difficulties surrounding a clear conception of the genesis, cell content, and etiology of focal lesions is illustrated in the formidable literature which has grown around researches into the various phases of the liver nodules in typhoid fever. Similar nodules occur in the paratyphoid infections of animals and it is probable that the same stimuli are operative in both typhoid and paratyphoid organisms. My limited histological contacts with the paratyphoid foci lead me to assume that the cells for which this group of bacteria are chemotactic are quite different from those dominating tuberculosis. They suggest endothelial cells overproduced, thrown off, and migrating to form nodules where bacilli have lodged and formed clumps so characteristic of typhoid and paratyphoid bacilli. This interpretation coincides with Mallory's early position. The final solution of this problem can be accelerated by a thorough study of the experimental and spontaneous paratyphoid lesions of animals, since in the human material many unknown and uncontrolled factors exist.[4]

SUMMARY

In the past half century pathologists have been inclined to refer all local cell gatherings to an active resistance on the part of the host. The concept of phagocytosis as a one-sided affair has dominated interpretations. All that we can postulate, however, is that certain bacteria and cell types have an affinity for each other. The outcome

[4] For a full discussion of this cell type see F. R. Sabin and C. A. Doan, *J. Exp. Med.*, 1926, **43**, p. 823.

of such affinity may be death of the host or suppression of the parasite, with various intermediate stages. Certain bacteria, as pointed out, associate themselves with cells of the connective and adenoid tissue which assume epithelioid type. Others like mouse septicemia and the Rickettsia have an affinity for vasçular endothelium. The typhoid-paratyphoid groups manifest a chemotactic affinity for endothelial leucocytes. The epithelioid type of cell reaction appears in tuberculosis and bovine paratuberculosis, in glanders, leprosy, and the induced *Brucella* and streptococcus disease of guinea-pigs. It is assumed that the reticulo-endothelial system is the source of these cells. Each group is the product of the specific action of the corresponding infectious agent and the expanding cytoplasm modified specifically, since there is no cross immunization among these diseases. They are the result either of direct contact of the bacteria with the progenitor cell or of diffusible metabolic products, or of products resulting from disintegration or dismantling of the bacteria. The behavior of the epithelioid cell depends upon genetic and acquired host factors, as well as upon virulence of the associated bacteria.

In spontaneous bovine tuberculosis the prompt central necrosis of the tubercle conceals the earliest phases. They are assumed to consist in the multiplication of the deposited bacilli followed by a prompt swelling of the cytoplasm of the local cells and subsequent necrosis. The outward spread and diminishing activity of the metabolic products of the tubercle bacilli lead to the epithelioid-cell mantle and finally the connective-tissue capsule. Multiplication of the cells in the tissues becoming epithelioid is limited or doubtful. Mitoses are not seen. In paratuberculosis the association of bacilli with the normal reticulo-endothelial system is observable and the presence of variously swollen cells ranging from fusiform to typical epithelioid cells can be seen in the progressive disease.

The specifically increasing resistance of the tissues as well as allergy is due to the discharge of diffusible products of the living bacilli and the continuous impact of these substances in minute doses upon the forerunners of the epithelioid cells throughout the system. When bacilli escape from a primary focus, the secondary foci are built under the influence of this increased resistance. They are smaller, with less

tendency to necrosis. Giant cells tend to appear in the secondary manifestations. They are indices of increased resistance in both tuberculosis and paratuberculosis.

From the evolutionary standpoint the gradual loss of diffusible, aggressive metabolic products by the invading parasite reduces the systemic effect and tends to localize and make chronic the disease process, provided the parasite at the same time gains in protective capacity. Host specialization rises and the host-parasite conflict becomes a purely cellular one. Paratuberculosis in cattle and leprosy in man are illustrations of these facts. The one is a progressive, fatal disease, the other one of extreme chronicity. In paratuberculosis the epithelioid cell dominates the field. There is neither necrosis nor fibrosis manifest.

The invasion of the primary epithelioid cell focus by polynuclear leucocytes does not occur in bovine tuberculosis or paratuberculosis. It does appear under conditions not understood in the induced bovine tuberculosis and *Brucella* disease in guinea-pigs and in glanders. There is no evidence that polynuclear leucocytes are involved in the initial stages of spontaneous tuberculosis.

In general the focal lesions in any infectious disease represent the conditions under which the bacterium can multiply and survive and eventually leave the host. Paradoxical as it may appear, these foci, owing to active cell immigrations and proliferations, have been considered the sites where the organisms are suppressed. It is, however, a fact that the same organisms are unable to survive elsewhere in the host, should they break away from the primary focus, unless similar foci are established. This dual nature of the focus as a place where the parasite multiplies and where at the same time it is being opposed by the host must be kept in mind in the interpretation of what can be seen by histological methods. The focus favors both host and parasite and stands for the compromise following interacting attempts at multiplication, on the one hand, and digestion on the other.

All bacteria must first exert some deleterious or stimulating effect on the tissues before a host reaction is set in motion. In the acute infectious disease the reaction promptly follows the immediate action or else the host succumbs. In the chronic affection both stages are drawn out, the action after a time merging into the reaction. The

obtruding action of the tubercle bacillus is necrosis, rapid, gradual, or merging into what appears like stimulation when virulence is low, or genetic resistance when it is high. By low virulence is meant that developed under host-parasite influence and not under artificial culture which is a different process not occurring under natural conditions. Necrosis may thus imply a direct toxic action of tubercle bacilli or a secondary effect following a too highly stimulated syncytium of epithelioid cells interfering with nutrition. In its mildest manifestations the factor underlying necrosis may induce a real stimulation of host-cell growth. All the factors entering into the inner workings of the tuberculous focus are in a state of flux or change, which it is at present impossible to visualize as a whole and which has led to such diverse views, each one exposing some fraction of the actual phenomenon. By defining the conditions, the scope, and the limitations of the problems in hand, all researches will eventually contribute to the final synthetic interpretation of the natural or spontaneous disease.

The idea presented in the foregoing pages is the stimulating activities of the tubercle bacillus. This may manifest itself in many grades of intensity, leading to the development of epithelioid cells, giant cells, the stimulation of fibroblasts, and possibly the direct production of necrosis in high concentration. The epithelioid cell is not related to the macrophage either as to origin or function. It is formed locally through contact of its progenitors with parasites or their products. It differs in these respects from polymorphs which are frankly phagocytic. Their activity amounts to a protection of the host generally, whereas the progenitors of the epithelioid and foreign body giant cells tend to perform this function locally. This idea is in contrast with the concept of phagocytosis which postulates prompt specific aggressiveness on the part of tissue cells. The idea implies, moreover, that immunity tends towards lesser and lesser susceptibility on the part of the cells instead of greater activity, and ending finally in the feeblest reaction the foreign body giant cell. The change following primary infection as manifested in secondary foci is assumed to be associated with antigenic and antibody products discharged from the primary foci which act upon the bacilli in transit and prepare the reticulo-endothelial cells for the secondary encounter. Finally, it

should be emphasized that differences in the histological structure of tuberculous lesions appearing more or less regularly may imply different races of bacilli as well as genetic or acquired differences in the resistance of individuals of the host species. A study of such variations among tubercle bacilli is still in its beginnings.

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GENETIC CONSTITUTION IN MICE AS DIFFERENTIATED BY TWO DISEASES, PSEUDORABIES AND MOUSE TYPHOID

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This paper* presents the preliminary phase of a study of the part heredity plays in an animal's ability to survive an inoculated virus disease, pseudorabies. Our choice of the genetically differentiated strains of mice included strains which had previously been differentiated for susceptibility and resistance to a bacterial disease, mouse typhoid. The contrast of the results attained for these two inoculated diseases furnishes evidence on the question of the composition of genetic constitution as it effects resistance to these diseases.

Material

Mouse strains which had been subject to differentiation by genetic technique were chosen to initiate this study. The differentiation which had been introduced had no known *a priori* relation to the disease chosen for study. Ancestors of one line had been selected on the basis of survival of an inoculated standard dose of *Salmonella aertrycke;* others had been mated brother and sister for several generations, with concurrent selection for the most vigorous and fertile progeny, etc. The selection and inbreeding have furnished strains which are rather homogeneous. This likeness of one animal to another reduces the

* The plan of this investigation called for an intensive study of the genetic constitution of mice in relation to their ability to survive inoculation of a virus disease, pseudorabies. Unfortunately circumstances beyond our control are such that the plan cannot be completed. The results here reported were to furnish the nucleus, the starting point, around which the other material was to be built.

variance between animals within a single strain. Sharp differentiation between strains is evidenced by known differences in their genetic constitutions and by their reactions to other agents.

The mouse colony in which these experiments were made was maintained under conditions as nearly uniform as possible. The diet was the same throughout the colony. It was chosen to contain as wide a variety of food materials as possible, the object being to supply each animal adequate amounts of the amino acids, carbohydrates, fats, vitamins, salts, and any other factors which are yet unknown but which undoubtedly play some part in nutrition. The mice had before them at all times a biscuit, which in itself was supposed to be an adequate diet, a seed mixture of 2 parts oats, 1 part each of sunflower, hemp and canary, and water. They were fed daily a cooked cereal composed of 1 part each of rolled oats, ground whole yellow corn, and ground whole wheat mixed with whole milk. Twice a week they received green alfalfa in its season, or cabbage in the winter.

The original outbreak of the pseudorabies virus which was used in these experiments occurred in a herd of cattle in Iowa. This disease was locally called "mad itch" from its main symptom, pruritus. Shope (1) obtained brain material directly from cows dead of the disease. Study of the pathology, etiology, species susceptibility, and immune relations have lead him to conclude that this outbreak is probably due to a previously described disease called pseudorabies. We are indebted to Dr. Shope for the inoculum which started these experiments. Our experiments have included several hundred mice, many with young. In no case have we had a case of the transmission of this disease due to contact. This agrees with the experiments of Aujeszky (2), Zwick and Zeller (3), Schmiedhoffer (4), and Shope (1) as all of these experimenters failed to get transmission through contact.

Pseudorabies virus is kept in stock by preserving in 50 per cent glycerine the brain of a rabbit dead of the disease. The virus used in mouse tests is always prepared when needed from freshly dead rabbit brain in the following way. The glycerinated material is washed thoroughly in sterile salt solution, weighed, ground to a smooth paste in a sterile mortar, and diluted with sterile salt solution so that each cubic centimeter contains 0.1 gram diseased rabbit brain. An adult rabbit of about 2000 grams is anesthetized, its skull is trephined, and

0.1 cc. of material is injected directly into the brain. The rabbit usually dies on the second day. The brain of the freshly dead rabbit is removed, part is preserved in 50 per cent glycerine. The rest is weighed, ground to a paste, and diluted to a 10 per cent suspension. From this stock suspension dilutions are made to contain the desired amounts used for inoculation of the test mice. Virus suspensions prepared in this way vary somewhat from one lot to the next, perhaps because of variations in the virus itself, or because of variations in reaction of different rabbit hosts. For that reason, each lot of virus must be titrated on a homogeneous group of mice to determine the dose required to kill a certain proportion of them. The amount of variation actually observed from experiment to experiment has been surprisingly little in the light of the variation ordinarily observed in biological material, table 1.

EXPERIMENTAL

Titrations have been made on four lots of rabbit virus material. The animals used for these titrations were white mice of Princeton Institute stock. Inoculations were intraperitoneal, of material contained in 0.5 cc. of suspensions.

The four titrations shown in table 1 show a fair degree of uniformity from experiment to experiment. The interval of time elapsing between them was first to second inoculation 3 months, second to the third 2 months, and third to fourth 3 months.

The homogeneity of the material tested by the χ^2 method leads to a χ^2 of 8.5 for the 16 test groups 10, 5, 1 mg. of brain and a P of 0.4 for the 9 degrees of freedom.

Experience with our other strains of mice shows that the Princeton Rockefeller Institute stock mice are very susceptible to the pseudorabies virus, more susceptible than any other group yet found. To obtain comparative susceptibility for these other strains it is consequently necessary to raise the dose above that which is lethal for the stock mice. Inoculations are intraperitoneal, the dose being 20 mg. of rabbit brain contained in 0.5 cc. of saline. The experiments in which strain differences are compared are all paired, strain for strain. The numbers of animals vary, however, as it is impossible to raise equal numbers of animals for test at a given time. The comparative

rates of survival for five different lines are shown below. Six other lines tested in small numbers showed intermediate rates of survival.

The proportion of mice surviving to the total number which were exposed to risk of death from the pseudorabies is shown in fig. 1.

TABLE 1

Rates of Survival of Princeton Rockefeller Institute Mice When Inoculated with Graded Amounts of the Brain of Rabbits Dead of Pseudorabies

Mg. of brain	First inoculum			Second inoculum			Third inoculum			Fourth inoculum		
	No. tested	No. survived	Per cent survived	No. tested	No. survived	Per cent survived	No. tested	No. survived	Per cent survived	No. tested	No. survived	Per cent survived
Above 40....	6	0	0	3	0	0	10	0	0			
30....	3	0	0	3	0	0	11	0	0			
25....							4	0	0			
20....	8	0	0	3	0	0	11	0	0	4	0	0
15....							4	0	0	4	0	0
10....	13	1	8	3	0	0	11	0	0	3	0	0
5....	10	1	10	3	0	0	8	0	0	4	1	25
1....	13	10	77	5	3	60	5	2	40	4	4	100
0.5....	10	8	80									
0.1....	13	13	100							2	2	100

TABLE 2

Rates of Survival of Genetically Different Strains of Mice When Inoculated with the Brain of Rabbits Dead of Pseudorabies

Strain	No. tested	No. survived	Per cent survived
S....................................	190	16	8.4 ± 1.4
W. f.................................	18	10	55.4 ± 7.9
Ba...................................	52	6	11.5 ± 3.0
Sch..................................	31	7	22.6 ± 5.1
sil..................................	176	92	52.2 ± 2.5

Strain S has a low survival rate when inoculated with pseudorabies. The Ba strain has a similar low rate. The sil strain has a relatively high rate of survival. That for the W. f. strain is also high but the small numbers available make the probable errors too large for this rate to have much significance. This strain furthermore has the real disadvantage of being genetically low in fertility and slow in breeding.

The sil strain was chosen for further study as having a genetic complex for survival on exposure to the pseudorabies virus, the S strain as a strain having intermediate survival value and the Princeton Institute stock for low survival value. Further differentiation of these stocks

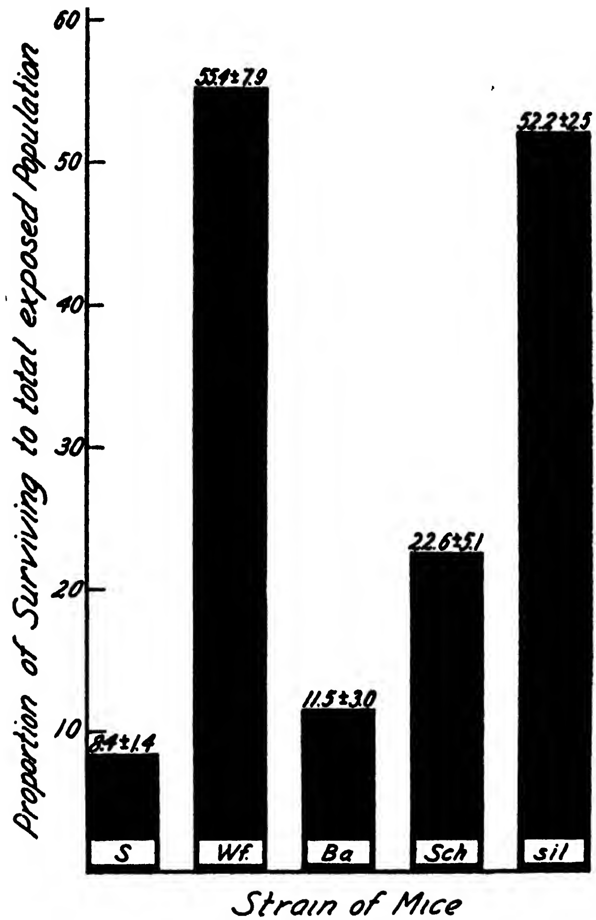

FIG. 1. Strain differences in rate of survival of mice to pseudorabies inoculations.

through proper matings is being studied as the greatest possible genetic differentiation has probably not been reached.

The significant differences in the susceptibility of these strains to the pseudorabies virus is of interest in connection with reports suggesting like strain differences for virus diseases affecting man (5).

For, while this material can scarcely be compared critically with that of man since study of human genetic material is notoriously difficult, the data which are here presented for mice show that genetic difference in the host population could well be one of the variables affecting the incidences and distribution of such human diseases as poliomyelitis (Aycock 6).

The genetic nature of the resistance to the pseudorabies virus is further brought out by crossing the S line with the sil line as seen below:

Parents: Strain S $\times$ strain sil

gave 59 F_1 progeny 14 of which survived test, a percentage survival of 23.7 ± 3.7.

The survival value of the hybrids is intermediate between the two parental lines and nearer the susceptible line. The F_1 survival rate is significantly different from both parental lines, however, the differences being $S - F_1 = 15.3 \pm 4.0$ and $\text{sil} - F_1 = 28.5 \pm 4.5$. The result could be interpreted as showing the genetic constitution of the sil line largely composed of recessive factors favoring resistance to pseudorabies whereas the S line contains a majority of dominant susceptibility factors, dominance being distinctly incomplete. The difference between the F_1 survival value and that of the mean between the two parental strains is not significant, however, so that the conclusion could also be drawn that the factors display no dominance, inheritance being of the blending type. Whichever conclusion is adopted the reader will notice the wide difference in the behavior of these F_1 hybrids with pseudorabies as contrasted with the behavior of like F_1 hybrids when exposed to mouse typhoid.

The significance of the differences in the survival rates of the various strains is indicated by the percentages and their conventional probable errors. It seems worth while to analyze the data from a slightly different viewpoint, by the χ^2 test, as the numbers are small. The χ^2's may be determined for the totals of the paired repeated tests or they may be determined for the individual tests summed and the *P*'s determined from the sum of the degrees of freedom. The second method is longer but indicates the consistency of the experimental results. The S and sil strains were early chosen for further breeding experiments. The data for these strains in table 2 are the total of 9 separated tests scattered over a period of 10 months during which time 4 different

brain emulsions were used for the source of virus. In every case the S strain shows a lower percentage of survival than does the sil strain.

Testing each experiment in a 4-fold table, summing the χ^2's and their degrees of freedom we obtain a χ^2 of 87.6 for the 9 degrees of freedom. The value of P derived from these results is consequently much less than 0.001. The strains S and sil would thus seem to be significantly differentiated in their susceptibility to the pseudorabies, the differences being manifest by consistent large differences in their reactions from experiment to experiment. The values of χ^2's for the totals of the test data on the different lines are shown tabled below.

These comparisons show the essential similarity between three of the five strains in their low survival of animals inoculated with pseudorabies. The other two strains, the sil and W. f., are significantly alike in showing a distinctly higher survival value than the first three. When the reader considers that each strain was produced by a process of selection which tended to make the animals within the strain like one another and that those strains were simply picked at random, these results take on added significance. A random bred stock would probably have both the resistant and the susceptible animals well mixed within it. The choice of any animal for test and the subsequent comparisons of that animal with others would consequently show all of the variation which is seen in the five lines here tabulated. The chance of picking out such heterogeneous individuals is furthermore approximately equality, 3 : 2, again emphasizing the significance of the genetic constitution in any animal population utilized for epidemiology or other studies in disease reactions.

Strains compared	χ^2	P
S and W. f.	33.4	<0.001
Ba	.4	0.51
Sch	5.7	0.02
sil	84.5	<0.001
W. f. and Ba	14.7	<0 001
Sch	5.5	0.12
sil	0.1	0.75
Ba and Sch	1.8	0.18
sil	27.2	<0.001
Sch and sil	9.3	0.002

This point is well exemplified by data obtained from 3 stocks of white mice obtained from different dealers. These mice were tested in parallel with those of our other experiments. In their reactions to the disease they exhibited the full range of variability which was noted in our resistant and susceptible lines. For many purposes such mixed lines are quite worthless, especially in experiments dealing with expensive animals in which the numbers must necessarily be small.

The data obviously cannot be interpreted as due to passive immunity transfer since no natural infection of normal by infected animals takes place in laboratory experiments.

Irwin (7), using the Danysz bacillus on rats, showed that resistance to the multiplication of these organisms inoculated in standard dose was to be attributed to a complex of genetic factors, some of which were practically dominant. Lambert (8) working with *Salmonella gallinarum* inoculated intraperitoneally in standard dose into 7-day old chicks, showed that the deaths from this cause could be reduced from 87 per cent to 9.4 per cent through the selection and breeding of survivors for five generations. Card and Roberts (9), in studying the disease reactions of chick strains to feeding a standard dose of *Salmonella pullorum,* showed that when the resistant strain was exposed to the disease 65.3 per cent survived whereas under like conditions only 27.5 per cent of the controls lived. Crosses showed that the resistance was practically dominant. Wright and Lewis (10), on different lines of guinea pigs, showed significant differences in their resistance to tuberculosis. In mice Tyzzer (11) observed a marked difference between reaction of waltzing mice, *Mus bactrianus*, and common mice, *Mus musculus*, to *B. piliformis*—an organism showing its effect chiefly in lesions of the liver and intestines. The writers have been able to show that this disease reaction is not linked in its inheritance to the waltzing factor. Pritchett's (12) experiments using stomach tube inoculations with mouse typhoid bacilli, Type II, show that two strains of mice were genetically differentiated from two others. Schott (13) by selective breeding derived strains of mice markedly different in their resistance to mouse typhoid, *S. aertrycke*. Genetically resistance appeared to be dominant and not sex linked. Webster (14) came to like views in studying lines of mice differentiated by progeny test into those which were susceptible and those which were resistant to *B. enteritidis*. Cole

(15) working with abortion in rabbits caused by inoculation of *B. abortus* into the dam was able to establish one group 100 per cent of which aborted and another group in which only 15 per cent aborted. Resistance to abortion is dominant in F_1. Furthermore, there is some indication that only a single pair of genes is concerned.

It will be noted that these cases for the inheritance of resistance or susceptibility to disease are all concerned with bacteria as the causative environmental agents. In plants inherited resistance to bacterial, fungus, and nematode worm infections have been noted. The data herein presented extend the observations of inherited resistance to include a disease classed in the virus group. The inheritance of the host resistance to this virus is distinctly different from that of those previously reported, the first generation hybrids being generally susceptible rather than resistant.

Differentiation of the Genetic Constitution of the S and Sil Mouse Strains by the Two Diseases, Pseudorabies and Mouse Typhoid

Genetic constitution could conceivably be either a single character which enabled the organism to resist a multiplicity of environmental agents or a composite of many independent characters each of which is capable of causing the individual to resist one environmental agent. The fortuitous combination of these characters could make animals resistant to many or to few such agents.

The philosophical implications which follow in the wake of these two hypotheses are markedly different. If constitution were a single character its inheritance would be expected to be simple. If on the other hand it were a composite of many individual characters then its inheritance need not be simple. The consequences of disease and their bearing on future diseases within a herd composed of individuals made up according to the first hypothetical constitution are very different from those in a herd composed of individuals with the second hypothetical constitution. The data of this paper seem to suggest that the constitution of mice is a composite made up of distinct characters some of which favor resistance to one environmental agent, others another.

The study of the genetic structure of constitution may perhaps best be approached by differentiating relatively pure and distinct lines within a species by matings designed to separate genetically distinct

lines and then studying these groups exhaustively from the viewpoint of their reaction to agents chosen because they are believed to be distinctly different.

The two lines S and sil were shown in the previous experiments to be distinct in their reactions to the pseudorabies virus. The S strain in a population of 190 showed the low survival rate of only 8.4 ± 1.4 per cent to this disease. The sil strain in the same experiment had a much higher survival, this rate being 52.2 ± 2.5 per cent in a population of 176. The F_1 hybrid's rate of survival was 23.7 ± 3.7 per cent in a population of 59. Many of those surviving the inoculation of the pseudorabies showed no symptoms of the disease, except that they became immune to a subsequent inoculation of a much larger amount of the virus which would otherwise have been surely lethal.

On the basis of survival, the sil stock would be considered much superior to the S stock. If constitution is a general over-all single character in inheritance the sil stock would be expected to show a higher survival value for any other pathological agent. If on the other hand constitution is made up like other general somatic characters we should expect it would be composed of many separately inherited entities.

It is possible to test this question since these two lines had previously been differentiated on the basis of their resistance to mouse typhoid, *Salmonella aertrycke* (13). The S line when inoculated with a standard dose of 5×10^4 organisms had 75.3 per cent of its individuals survive. The sil strain on the other hand had no mouse survive this dose. Crosses between these lines showed that the resistance of the S line was transmitted through both males and females. The survival rate of the F_1 crosses was 62.6 per cent. The typhoid disease behaved differently from the pseudorabies disease in the fact that all animals inoculated showed distinct symptoms of the disease. None escaped showing some traces of its effect. A test through the use of double matings (24) has indicated these differences to be genetic in origin.

The significance of these differences may be tested by their χ^2 values. In the pseudorabies experiments there were 190 mice of the S line tested, 174 of which died and 16 survived. In the same experiment the sil strain had 176 mice tested, 84 of which died and 92 survived. The F_1 cross between the S and sil lines had 59 progeny of which 45 died in

test and 14 survived. In the previous generations the test for the survival value of these three groups to an inoculation of a standard dose of *Salmonella aertrycke* organisms showed that of 105 tested in the S line 26 died and 79 lived; of 108 individuals tested in the sil line all died; and of 187 F_1's tested 70 died and 117 survived. The χ^2 and P values for the different combinations are shown tabled below.

	Classes compared	χ^2	P
Pseudorabies	S with sil	84.5	<0.001
	S with F_1 (S × sil)	10.0	0.002
	sil with F_1 (S × sil)	14.5	<0.001
Pseudorabies rates compared with *Salmonella aertrycke* rates		225.6	<0.001
Salmonella aertrycke	S with sil	30.5	<0.001
	S with F_1 (S × sil)	38.5	<0.001
	sil with F_1 (S × sil)	110.8	<0.001

The survivorship curves shown in fig. 2 bear out the same interpretation. The solid line represents the per cent of the sil strain surviving plotted against days after inoculation. The dot and dash line shows the same information for the S strain. The F_1 hybrids survivorship curves are represented by the dotted lines. The plots are made on the semi-logarithmic scale so that equal rates are visible to the eye. The pseudorabies graph is quite distinct from that of the mouse typhoid in a number of particulars. There is first a definite incubation period. When deaths commence on the third day the survival line falls in very nearly straight lines from the initial origin of 100 per cent living animals throughout the cycle of the disease, when deaths cease nearly as sharply as they began. The cycle of the disease consists of a 3 day incubation period followed by 6 days when deaths are taking place. It will be noted that the S animals die at a much faster rate than the sil strain. The F_1 rate is approximately intermediate between the two parental strains.

The mouse typhoid curves present a markedly different picture. Deaths commence on the second day with a slow but constantly increasing rate. The sil and the S strain have now reversed their rates of death from those seen in the pseudorabies disease. All of the sil animals die off rapidly, the survivorship curve reaching zero on the

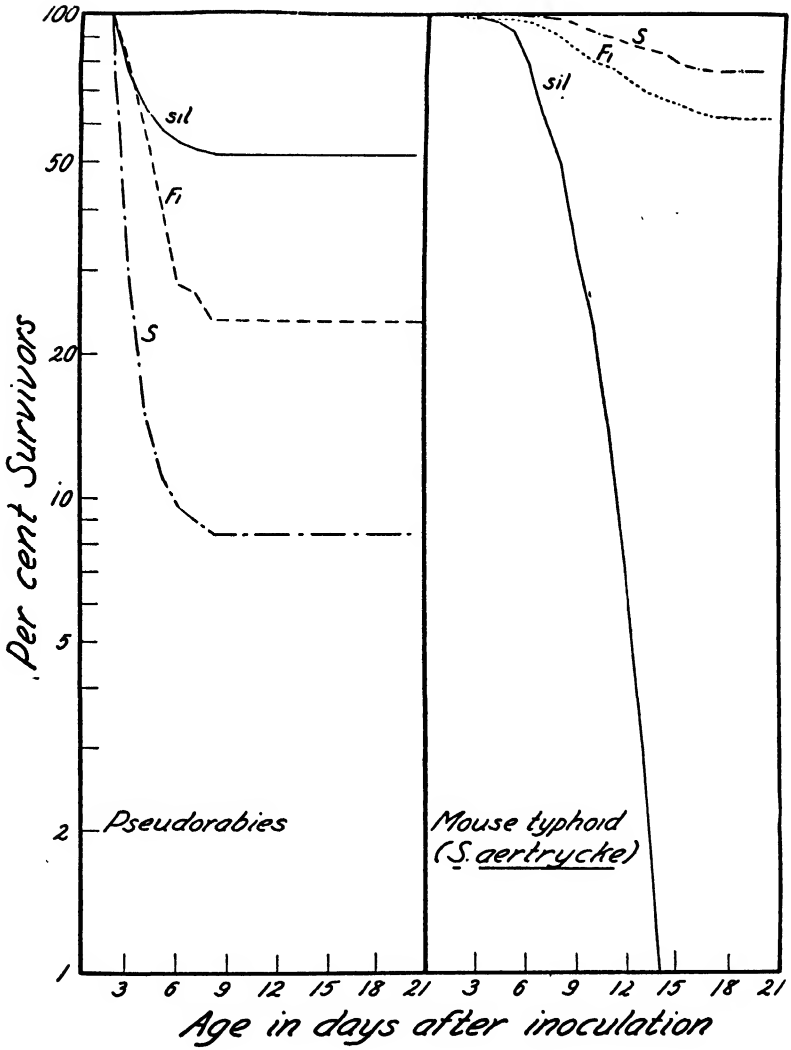

FIG. 2. Survivorship curves of the S and sil strain and their F_1 hybrids for pseudorabies and mouse typhoid.

14th day. The S strain shows a slow rate of death and a high final survival value. The F_1 hybrids show somewhat less survival than the S parents and a markedly greater survival than the sil stock. The duration of the disease is distinctly longer with the mouse typhoid than with the pseudorabies with all strains.

These data show that the two lines of mice, S and sil are distinctly different in their reactions to pseudorabies virus on the one hand and the mouse typhoid organism, *Salmonella aertrycke*, on the other. The fundamental basis of these differences has been shown to be the genetic constitutions of these two strains. A genetic complex which is favorable to the survival of a strain when exposed to one pathological agent is here shown to favor susceptibility to another agent. The hybrids react like the S parents, showing a marked difference in dominance of the genes comprising the constitutions of these two lines.

The genetic constitution for disease resistance or susceptibility to these diseases is shown by the data to be a composite of separate genes capable of causing the individual to resist one or sometimes more environmental agents, the fortuitous combination of these separate factors making animals resistant to many or few such agents. The conclusion to be derived from these data is thus in conformity with the extensive data on host resistance in plants (16, 17, 18, 19, 20).

The work of Lambert and Roberts seems to point to a similar conclusion for the diseases of fowl cholera and bacillary white diarrhea in the domestic fowl. Certain of the results of Webster (21, 22, 23),—on widespread dissemination,—have been interpreted otherwise, due, we believe, to the habit of many in reading summaries rather than the whole original paper. One of the difficulties lies in the use of the words specific and non-specific factors for resistance since as the writer has pointed out (24) these words have distinctly different meanings depending on what scientific vocabulary the author and reader are using. In Webster's usage specific factors are used in the immunological sense of the specific immune bodies known to be developed against a given disease organism or foreign antigen. These bodies are, of course, no more specific than the genes, the specific factors, of inheritance some of which are responsible for the transmission of disease resistance. The experimental results were as follows. Mice were inoculated by os with 6 strains of mouse typhoid recognized as

distinct. The 60-day survivors of each group were given a fixed dose of bacteria of one epidemic mouse typhoid strain, M. T. II. The deaths for the six groups after the second inoculation were related more directly ($r = 0.9$) to the virulence of the strain of bacillus originally administered than to the antigenic similarity of this first strain to the second strain employed ($r = 0.09$). Webster has thus made it possible, speaking in the vocabulary of the immunologist, "that the resistance mechanism of the host contains important non-specific factors which vary in degree with the individual mice." But this use of the term "non-specific" cannot be transposed into the science of genetics as some readers have done. Data are lacking to enable us to determine whether the resistance could be accounted for by the same or different genes. A like situation exists with regard to resistance to mercuric chloride of survivors of a previous mouse typhoid inoculation, although here there is a wide experimental variation observed in the material (21, 23).

SUMMARY

The data herein presented show that the ability to survive a given inoculation of the virus of pseudorabies is markedly influenced by the genetic constitution of the animal. Susceptibility shows some tendency to be dominant in the F_1 cross. Comparison of a line which was resistant to pseudorabies and another which was susceptible for their respective resistances to another disease, mouse typhoid, showed their reactions to this second disease completely reversed. The F_1 cross for this second disease now show susceptibility largely recessive. These facts lead to the conclusion that genetic constitution as it is related to resistance to these diseases is perhaps best regarded as a composite of several distinct genes, some favoring resistance or susceptibility to one environmental agent and some another.

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A GENETIC TECHNIQUE FOR DIFFERENTIATING BETWEEN ACQUIRED AND GENETIC IMMUNITY

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Two hypotheses have been suggested to account for increased immunity in progeny of survivors of a disease. One hypothesis is that the progeny inherit genetic factors which make their parents resistant. The other hypothesis is that the progeny acquire an immunity either active or passive.

The genetic hypothesis is based on the fact that a large random bred population of animals has wide variability for all characteristics thus far measured. Differentiation of the population, by a disease, into animals which survive and animals which die might separate two groups differing in their inherited factors for resistance to the disease. Survivors bred together would then produce progeny which contained proportionately more inherited factors for resistance than the initial population. If animals from this later generation were separated into survivors and non-survivors by exposure to the disease a higher proportion of survivors would be found. Breeding these survivors together would again result in selection for resistant factors. This process, continued for many generations should produce a steadily declining death rate, each generation being of a more favorable genetic constitution than its predecessor. A decline in susceptibility due to selection of this type would take place in a quite predictable manner, with curves assuming rather definite forms. The work of Irwin (1), Lambert (2), Roberts (3), Schott (4), and Webster (5) furnish survival curves illustrating the expected consequences of this hypothesis.

The hypothesis of acquired immunity reasons from the fact that

animals which survive a disease develop an active immunity of irregular duration. Some survivors retain a latent infection which is thought to protect them by keeping up an active immunity. These carriers may spread sublethal infection to their progeny, thereby inducing active immunity in the progeny. Evidence indicates that passive transfer of immunity from dam to progeny may take place, but males are not able to transmit passive immunity to their offspring.

A method of determining which of these hypotheses is correct is significant to the study of any host-disease relationship and is of particular importance in testing inheritance as a factor in disease resistance.

The difficulties which beset the path of those who attempt to differentiate between these hypotheses are well illustrated by Schott's study of the susceptibility of mice from selected strains to *Salmonella aertrycke* inoculation. A review of these experiments is helpful in clarifying the problem and the experimental conditions which must be met. A standard dose of 5×10^4 organisms per mouse injected intraperitoneally caused 82.3 per cent mortality in one mouse strain. The survivors were mated and their progeny were tested with the standard dose. This process was continued for six generations with death rates as follows:—F_1 64.5, F_2 45.8, F_3 39.8, F_4 36.3, F_5 32.6, F_6 24.7 per cent. Each decline in percentage of deaths is significant. This mouse strain has been designated S. The fact that the F_1 generation of the S strain was more resistant than the P_1 can be explained by either hypothesis i.e., either that the survivors have in their germ plasm constitutional factors for resistance which are inherited by the progeny, or that progeny of survivors acquire immunity from contact with their parents. The continued decline in death rates in later generations favors the genetic hypothesis. A steady increase in survival value would be expected from continued selection of genetic factors for resistance, but it is not the result which would be expected from acquired immunity. The latter hypothesis does not explain why the death rate should decline at all after the first generation.

Inoculations of the same standard dose at the same times into mice of another strain, called sil, caused 100 per cent mortality in every test. Females from this highly susceptible sil strain mated to male survivors from the S strain produced progeny whose death rate at test was 37.5 per cent, showing that these progeny were definitely more

resistant to *Salmonella aertrycke* inoculation than their mothers. This can be explained easily on the hypothesis that genetic factors for resistance were contributed by the father's germ plasm. The other hypothesis is less satisfactory here. It assumes that the surviving males carry a latent infection and induce an acquired immunity in their progeny by transmitting to them a sublethal attack of the disease, unless per chance we choose to enter a dilemma by assuming that the organisms were generally attenuated by their residence in the male mouse.* If this were the case, the susceptible mothers should have taken the disease and either died or become immune. They did not die, and were mated to susceptible sil males for second litters. If transfer of acquired immunity were the correct hypothesis, these second litters should have been protected as well as the first. If the genetic hypothesis were correct, the second litters should have been 100 per cent susceptible. When tested, the mice from the second litters had a death rate of 100 per cent.

If passive immunity transfer has any real significance for the problem under discussion, the progeny of surviving S females and susceptible sil males would be expected to have greater resistance than progeny of sil females and surviving S males, since in the first case the young might receive immunity from their mothers by passive transfer. On a series of seventeen and twenty-seven mice, the death rates of these two groups were the same 35.4 and 37.0 per cent.

It is of importance to note that those who emphasize the significance of immunity transfer in accounting for the transmission of resistance from one generation to the next must necessarily assume the existence of infection in the selected stock. The question naturally arises are all investigators in this field such uniformly poor technicians? The evident care with which experiments of Webster's (5, 6) and Manresa (7) have been designed and conducted to avoid such infection make such an assumption distinctly difficult to accept.

* If this assumption is made to account for resistance of the F_1 how, on these grounds, can the hypothecated infection or immunization of these animals with the postulated attenuated organisms take place where the mothers constantly living with them uniformly develop no immunity? The alteration of virulence of organisms of this group under conditions as of the present test is furthermore contrary to the observed results of Webster's (6) numerous experiments.

Double mating technique

Albino ♂

Resistant to mouse typhoid.

and

Silver ♂

Susceptible to mouse typhoid.

A single silver ♀ susceptible to mouse typhoid mated in same oestrus to both males.

Gives a mixed litter composed of

F_1 Hybrids
black in coat color

and

Pure Silvers
silver in coat color

in same litter.

Mouse typhoid bacilli in dose used kill

53% F_1 progeny.

100% silver progeny.

FIG. 1. Technique utilized in obtaining progeny of two different genetic constitutions in same litter.

The evidence thus far presented possibly favors the hypothesis of genetic immunity rather than that of acquired immunity but it is not definite enough to convince those who hold the latter hypothesis. We suggest a technique applicable to a wide variety of diseases which seems to be particularly applicable to this problem.

The S and sil strains differ in their genetic factors for coat color, so that it is possible to distinguish between S, sil and hybrids. A sil female mated in the same oestrus period to two males, one sil and one S, may have sil and hybrid progeny in the same litter. Environmental conditions are common to all animals of a litter, each having the same opportunity to receive immune bodies from the mother and to receive latent infection from the parents. According to the genetic hypothesis the sil progeny should be 100 per cent susceptible and the hybrids should show resistance. According to the acquired immunity hypothesis the sil and hybrid litter mates should be equally susceptible. Figure 1 shows the technique graphically.

TABLE 1

Survival in Days of Mice Inoculated with Salmonella aertrycke

	3 to 6	6 to 8	8 to 10	10 to 12	12 to 14	14 to 16	16 to 18	18 to 20	20 to 22	Lived	Total
Sil × sil single mating	7	31	31	20	11	4					104
Sil × sil double mating......		4	9	5	2	1					21
S × sil double mating			1	2	3	1	1			7	15
S × sil single mating........	5	9	16	10	15	7	7		1	117	187

Six successful double matings were obtained, with twenty-one sil progeny and fifteen hybrids. The young mice were weaned at the age of four weeks and at seven weeks they were shipped by express to Ames, Iowa so that the inoculation with *Salmonella aertrycke* could be made under conditions comparable with those of the previous work. This 1200 mile trip undoubtedly tended to make all the mice more susceptible to disease. At sixty days of age the mice were inoculated with the standard dose. The death rates were 100 per cent for the sil mice and 53.3 per cent for the hybrids. This difference is statistically significant since the χ^2 value is 12.2 with a P of 0.0005.

Table 1 shows that in time of death as well as in proportion of deaths,

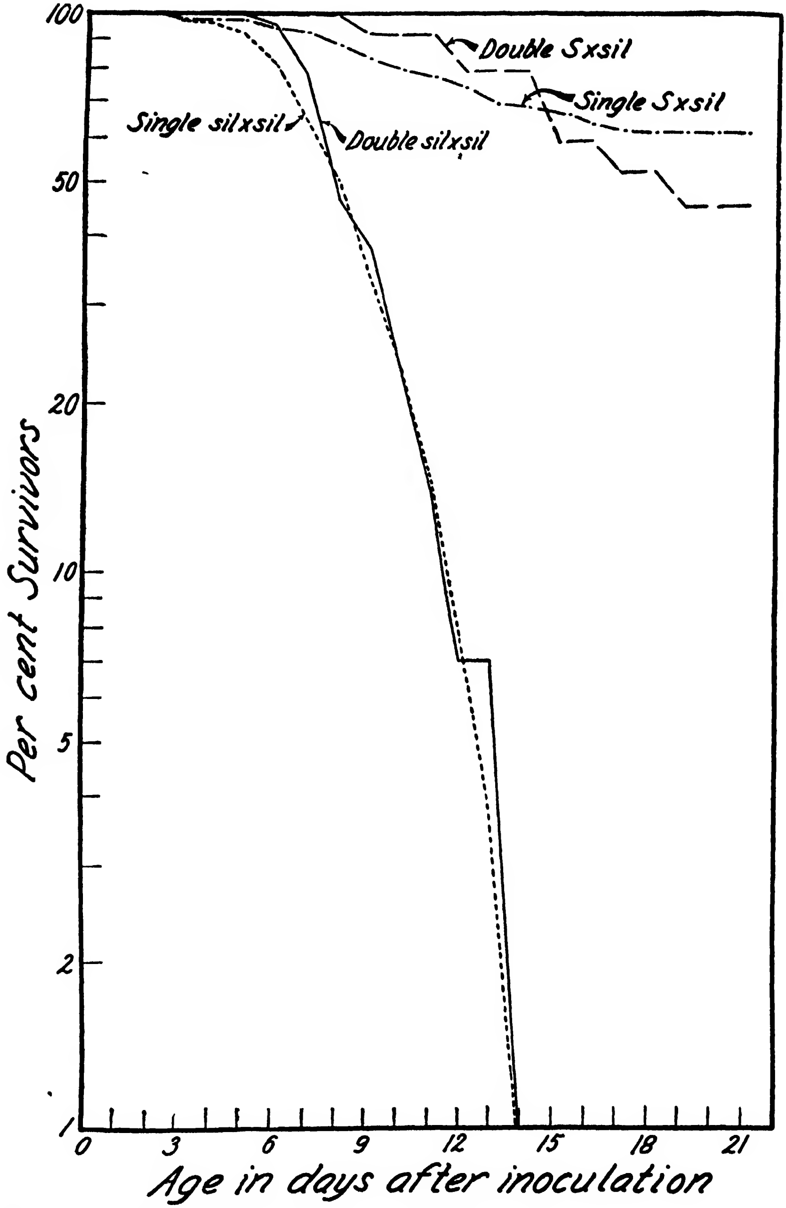

FIG. 2. Survivorship plotted against age after inoculation for mice derived from three different types of matings sil × sil, S × sil and double mated sil females mated to both sil and S males.

mice from double matings resemble mice of the same parentage from single matings. Figure 2 shows the percentage of survivors of the initial population exposed to risk for each day of age after the inoculation. These data are plotted on the arith-log grid making it possible to compare the rates of survival directly line for line. It is strikingly evident that the survivorship lines of the pure sil lines in the single matings and in the double matings are entirely similar. Likewise the F_1 progeny of the S × sil parents whether from single or double matings are equally alike in their survivorship curves. The sil curves and the F_1 S × sil curves are distinctly different. Is it not likely that the similarities and contrasts in these two sets of data represent a real phenomena, that animals which survived mouse typhoid inoculation are immune because of their genetic constitution and not because of acquired immunity?*

SUMMARY

Double mating is suggested as a genetic technique for distinguishing between acquired and inherited immunity. This technique seems to favor the hypothesis that resistance to *Salmonella aertrycke* inoculation in a selected strain of mice is due to concentration of genetic factors for resistance and not to transfer of acquired passive or active immunity.

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* Statistically the data of this paper are large enough to be significant. The progeny within each of the six litters reacted in the same manner again strengthening the belief that the observed differences are real. While the writers would have preferred larger numbers circumstances beyond our control are such that this work must be brought to a close at this stage. As it stands this paper is to be regarded as a description of a technique applicable to the solution of a wide variety of disease problems and not as a final answer to the problem of what causes lie behind the increased resistance of successive generations of animals derived from selected surviving parents.

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CONFORMATION OF THE COW AS RELATED TO MILK SECRETION, JERSEY REGISTRY OF MERIT

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Two major methods of ascertaining the probable milk secretion of a dairy cow have been suggested. There are the cow's conformation or physical appearance and her heredity or the ancestral record of milk secretion contained in her pedigree. Both methods are applicable to cows which are too young to yield milk, or whose own records are unknown or believed untrustworthy. Both methods have been cursed by having much written about them when little or no scientific information was available to determine validity of the good attributed to the systems. The conformation method has been especially tormented by the fact that judging of dairy cows became a sporting proposition to be played at fairs, and the scale of points became the rules by which the game was to be played even though conformation might have no relation to production. In view of the lack of objective evidence on these problems and the prominence given judging in animal husbandry teaching (16, 17), the writer (7, 8, 9, 10, 11, 12, 13, 14) has since 1914 made an effort to determine the real merits of these two methods, conformation and heredity, in relation to milk secretion, and to furnish comparative numerical data by which the particular contribution of each item to the cow's milk secretion could be evaluated.

In the long run, constructive breeding must have as its ideal the production of an animal or strain which utilises its food to manufacture, most economically, products for human consumption. In beef cattle the case is perhaps rather simple, since the conformation of the animal has such an immediate and direct relation to the amount and character of the beef taken from the carcass. Hogs are possibly

somewhat more difficult. Text-books inform us that the ideal type carries a short, compact body. Careful carcass comparisons have shown that such a type is not the true ideal, since it does not give the maximum of cuts desired for human food.[1] The case is even more difficult for dairy cattle, since the characters leading to a large flow of milk are but improperly understood and are rather well hidden. Furthermore, the dairy-cattle breeder uses type in two quite distinct ways. If he is selecting dairy cattle for his own herd he will frequently purchase cows of unknown age on the theory that there is a direct relation of conformation and production without regard to age. Historically this is the way in which conformation was first used to predict production. The show-ring use of type is quite different, as cows are grouped in age classes thereby correcting for age. Both problems are important and will receive consideration. These questions may be approached through the use of the data on the American Jersey cattle. These data include the following measurements on the bull's or cow's type: weight, height at withers, heart girth, paunch girth, width at hips, body length, and rump length. Fig. 1 shows the relative position of the measurements taken. About 300 bulls and 6000 cows were measured. These represented animals from fifteen different states distributed quite generally throughout the United States. Two men took all of the measurements; one man, Mr. Randolph, took all but about 800. Both men were capable, experienced dairy-cattle men. The measurements were taken in 1922. The ages, parentage, etc., of these animals are known. The analysis of the material and the first written draft were made by 1925. Of these cows, 840 have Registry of Merit records for production. The relative influence of the different points of type on milk production may be best shown in illustrated form. Fig. 2 shows these results in an ordinary bar diagram. The length of the bar shows the relative influence of the given item of conformation on the production of the cow, *i.e.* it represents the value of the correlation coefficient between the type measurement and the cow's production record.

The most striking feature of Fig. 2 is the marked influence of type on milk production or butter-fat production with an almost complete

[1] Wentworth, E. N. and Ellinger, T. W. 1925. Long-bodied *v.* short-bodied hogs. *Armour's Live Stock Bureau,* 6, 9, December.

FIG. 1. Photographs of Jersey Cows showing the points actually used in making the measurements studied herein.

lack of effect on the butter-fat percentage. The next most striking feature is the preponderance of the influence of weight[1] on milk and butter-fat production as contrasted with the other elements of type. The other measurements making up type appear to have equal influence on the milk production or butter-fat yield of the cow. The data for these conclusions are shown in Table I.

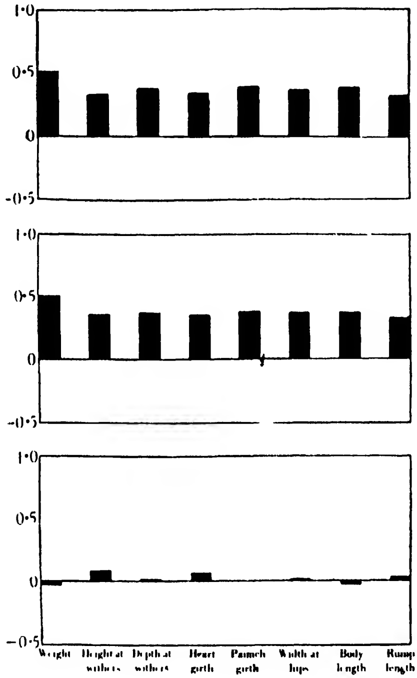

FIG. 2. Relation between conformation and production of Jersey Registry of Merit cows, all ages. The height of the bars represents the value of the parts of conformation as a means of indicating production. Weight of the cow is clearly most important in indicating milk or butter-fat production. The other parts of conformation predict the cow's production to about equal degree. Conformation shows little about the butter-fat percentage of the cow's milk.

The body measurements—height at withers, depth at withers, heart girth, paunch girth, width at hips, body length, and rump length—

[1] The influence of weight on production has been frequently noted (2, 3, 13, 23, etc.), but its effect on milk secretion compared to that of other measures of conformation has been analysed for but one other breed (10, 12).

are, broadly speaking, dependent upon the skeletal development of the cow. They are in a sense bone measurements, for it is the growth of the bones which determines their lengths. Weight on the other hand takes into account not only the size of these bones but also the amount of flesh which is on them. A difference is thus to be expected between the relation of weight to production and of the type measurements—depending only upon bone growth—to production. Such a difference

TABLE I

Average Size, Variation in Size, and Correlation between Measurements of Conformation and Production in 840 Jersey Registry of Merit Cows, Old Requirement

	Average	Standard deviation	Correlation with*		
			Milk yield	Butter-fat %	Butter-fat
Age	4.29 yrs.	2.15 yrs.	0.281	−0.121	0.241
Weight	804 lb.	132 lb.	0.510	−0.019	0.506
Height at withers	46.9 in.	1.9 in.	0.326	0.078	0.360
Depth at withers	26.2 in.	1.8 in.	0.373	0.005	0.374
Heart girth	68.1 in.	4.1 in.	0.339	0.061	0.354
Paunch girth	82.6 in.	6.1 in.	0.388	0.000	0.382
Width at hips	19.5 in.	1.7 in.	0.357	0.008	0.351
Body length	35.7 in.	2.8 in.	0.376	−0.024	0.375
Rump length	19.9 in.	1.5 in.	0.321	0.324	0.327
Milk yield (365 days)	9085 lb.	2455 lb.	—	−0.231	0.922
Butter-fat %	5.49	0.558	−0.231	—	0.138
Butter-fat	495 lb.	133 lb.	0.922	0.138	—

* The height of the bars in Fig. 2 is equal to the correlation coefficients; the scale is the correlation scale.

actually appears in these measurements. It would seem that not only the size of the cow as determined by her skeleton, but also the degree of fleshing which she carries, has a pronounced influence on her productive capacity.

Our experience teaches us that a cow grows symmetrically. From this fact it would be expected that all measurements depending on skeletal size would have similar relations to production. The results agree with this hypothesis, since for the bone measurements—height at withers, depth at withers, heart girth, paunch girth, width at hips,

body length, and rump length—no significant difference is found in their relation to the milk production or butter-fat production of the cow. In other words, if measurements of only one of these items were made, practically as good an indication of the cow's production would be obtained as if measurements were taken of all the parts of conformation. The case for weight is somewhat different. Besides the skeletal measurements weight also measures the fleshing of the cow. Weight should, consequently, show a different relation to the cow's production than the seven skeletal measurements. Such actually proves to be the case. Weight, as shown by Fig. 2, clearly is a better indication of the cow's production than any single one of the skeletal measurements.

The results also show that the probable butter-fat percentage of the cow is relatively hidden in so far as any of these measurements are concerned. It is clearly impossible to predict satisfactorily the probable butter-fat percentage of the cow from these measurements of type.

The data for the measurements of type are arranged in groups according to the butter-fat production of the cows. These groups are: first, cows producing under 400 lb.; second, cows producing 400–600 lb.; third, cows producing 600–800 lb.; and fourth, cows whose production is above 800 lb. These results are shown in Table II under A. The data are also arranged according to milk yield. The measurements are in groups: under 5000 lb., 5000–10,000 lb., 10,000–15,000 lb., and 15,000 lb. or above. These groups are given in Table II under B. The data are further arranged in groups according to the butter-fat percentage: under 4 per cent., 4–5 per cent., 5–6 per cent., 6–7 per cent., and 7 per cent. or above. The average measurements for each item of conformation are shown for these groups in Table II under C.

Table II shows that there is a fairly steady increase for each of the size measurements as the butter-fat yield or milk yield of the cow increases. In other words, the larger the yield, the larger the size of the cow. This same relation does not hold for the butter-fat percentage. Cows of the same size for any of the different type measurements may differ markedly in their butter-fat percentages. The butter-fat percentage is not influenced significantly by the size of the cow.

Table II A for butter-fat may be expressed in another way. For each 100 lb. increase in butter-fat it is found that there is an average increase in weight of 50 lb., an average increase in height at withers of 0.5 in., depth at withers 0.5 in., heart girth 1.1 in., paunch girth 1.8 in.,

TABLE II

Average Production and Size Measurements for Jersey Cows

A. Arranged according to the butter-fat production of these cows. All ages

Butter-fat	Number	Butter-fat	Weight	Height at withers	Depth at withers	Heart girth	Paunch girth	Width at hips	Body length	Rump length
lb.		lb	lb.	in	in	in.	in.	in.	in.	in.
Under 400	215	351.8	722.5	46.0	25.4	66.4	79.8	18.8	34.5	19.3
400–600	463	486.5	806.0	46.9	26.2	67.9	82.6	19.5	35.7	19.9
600–800	135	680.3	896.0	47.8	27.1	70.4	86.0	20.4	37.2	20.7
Above 800	27	866.3	971.5	48.7	27.8	70.1	88.3	20.8	38.9	21.0

B. Arranged according to the milk production of these cows. All ages

Milk yield	Number	Milk yield	Weight	Height at withers	Depth at withers	Heart girth	Paunch girth	Width at hips	Body length	Rump length
lb.		lb.	lb.	in.	in.	in.	in.	in.	in.	in.
Under 5000	12	4,625	637.5	45.8	24.1	65.1	76.8	18.5	33.9	19.1
5,000–10,000	569	7,815	770.5	46.5	25.8	67.2	81.3	19.2	35.2	19.6
10,000–15,000	236	11,695	880.5	47.6	26.9	69.7	85.3	20.2	36.9	20.5
Above 15,000	23	16,010	951.0	48.1	27.7	71.4	88.6	21.0	38.3	21.0

C. Arranged according to the butter-fat percentage of these cows. All ages

Butter-fat	Number	Butter-fat	Weight	Height at withers	Depth at withers	Heart girth	Paunch girth	Width at hips	Body length	Rump length
%		%	lb.	in.	in.	in.	in.	in.	in.	in.
Under 4	2	3.95	900.0	47.5	27.5	70.0	83.0	20.0	36.5	20.0
4–5	165	4.75	800.5	46.6	26.1	67.3	82.2	19.4	35.6	19.8
5–6	523	5.49	810.5	46.9	26.3	68.4	82.9	19.6	35.7	20.0
6–7	146	6.32	785.0	47.0	26.1	67.8	82.0	19.4	35.4	19.9
Above 7	4	7.25	837.5	47.5	27.0	68.0	81.0	19.3	34.5	19.3

width at hips 0.4 in., body length 0.8 in., and rump length 0.4 in. The results for milk yield, Table II B are: an increase of 1000 lb. of milk results in an average increase in weight of 28 lb., height at withers 0.3 in., depth at withers 0.3 in., heart girth 0.6 in., paunch girth 1.0 in.,

width at hips 0.2 in., body length 0.4 in., and rump length 0.2 in. The results for the butter-fat percentage, Table II C, are extremely irregular. In fact there is no significant relation between the butter-fat percentage and the size measurements of the cow.

The results may be arranged in a different form, a form which throws some light on the relation conformation bears to possible economy of production. To facilitate making these comparisons the data may be presented in equation form. These equations are given below. They apply strictly only to cows capable of making the Registry of Merit and possibly only to these particular animals, since the Registry of Merit requirements have been changed.

Butter-fat = 86.3 + 0.509 weight.
Butter-fat = 24.84 height at withers − 668.9.
Butter-fat = 27.33 depth at withers − 218.4.
Butter-fat = 11.51 heart girth − 288.1.
Butter-fat = 8.28 paunch girth − 188.5.
Butter-fat = 27.74 width at hips − 46.3.
Butter-fat = 17.79 body length − 139.0.
Butter-fat = 28.29 rump length − 68.8.

Milk yield = 1470 + 9.47 weight.
Milk yield = 414 height at withers − 10,333.
Milk yield = 502 depth at withers − 4071.
Milk yield = 203 heart girth − 4724.
Milk yield = 155 paunch girth − 3748.
Milk yield = 521 width at hips − 1084.
Milk yield = 329 body length − 2647.
Milk yield = 513 rump length − 1154.

Butter-fat % = 5.56 − 0.0000819 weight.
Butter-fat % = 4.44 + 0.0224 height at withers.
Butter-fat % = 5.49 + 0.000150 depth at withers.
Butter-fat % = 4.92 + 0.00831 heart girth.
Butter-fat % = 5.49 + 0.0 paunch girth.
Butter-fat % = 5.44 + 0.00259 width at hips.
Butter-fat % = 5.66 − 0.00484 body length.
Butter-fat % = 5.25 + 0.0122 rump length.

The prediction for the butter-fat yield of the cow as indicated by weight is as noted above, 86.3 + 0.509 weight. The probable butter-fat yield of an 800 lb. cow would be $86.3 + 0.509 \times 800 = 86.3 +$

407.2 = 493.5 lb. of butter-fat. Similarly for a cow weighing 900 lb. the probable production would be $86.3 + 0.509 \times 900 = 86.3 + 459.1 = 544.4$ lb. of butter-fat. The difference between these butter-fat yields is equal to $544.4 - 493.5 = 50.9$ lb. It is possible, therefore, to get the difference in production between cows as simply the difference in size times the constant given in the proper equation. That is, two cows which differ by 100 lb. in weight, would probably differ in their butter-fat yield by an amount equivalent to 0.509×100 or 50.9 lb. of fat. These equations therefore lead to the following interesting conclusions. A difference of 100 lb. in weight between cows results, on the average, in an increase in the butter-fat yield for the larger cow of 50.9 lb., 1 in. in height at withers 24.8 lb., 1 in. in depth at withers 27.3 lb., 1 in. in heart girth 11.5 lb., 1 in. in paunch girth 8.3 lb., 1 in. in width at hips 27.7 lb., 1 in. in body length 17.8 lb., 1 in. in rump length 28.3 lb.

On the same basis a difference of 100 lb. in weight results in an average increase in production of 947 lb. of milk in favour of the larger cows. A difference of 1 in. in size favours a greater production in the larger cow of 414 lb. for height at withers, 502 lb. for depth at withers, 203 lb. for heart girth, 155 lb. for paunch girth, 521 lb. for width at hips, 329 lb. for body length, and 513 lb. for rump length.

The influence of any of these measurements on butter-fat percentages is so slight that the equations all lead simply to the average value of the breed's butter-fat percentage whatever the size of the cow may be.

Perhaps the most interesting analogy results from a comparison of what the cow of largest size for any given point of type would be expected to give for her butter-fat or milk yield as contrasted with the production of the smallest cow for this point of type. The smallest cows of milk-producing age have something like the following measurements: weight 500 lb., height at withers 41 in., depth at withers 19 in., heart girth 55 in., paunch girth 59 in., width at hips 14 in., body length 25 in., and rump length 15 in. The largest cows have the following measurements: 1350 lb. weight, 53 in. height at withers, 32 in. depth at withers, 82 in. heart girth, 100 in. paunch girth, 25 in. width at hips, 43 in. body length, and 24 in. rump length. The difference in butter-fat yield between these two extremes would be for weight 433

lb., height at withers 298 lb., depth at withers 354 lb., heart girth 311 lb., paunch girth 340 lb., width at hips 305 lb., body length 320 lb., rump length 255 lb. For milk yield the difference in production

TABLE III

Average Butter-Fat Production and Weight, Milk Yield, and Butter-Fat Percentage for Jersey Registry of Merit Cows. All Ages

Arranged by butter-fat yield

Butter-fat	Actual weight			Estimated weight		
	Number of cows	Average butter-fat	Average weight	Number of cows	Average butter-fat	Average weight
lb.		lb.	lb.		lb.	lb.
Under 400	516	346	825	4268	344	806
400–600	683	479	908	5274	476	886
600–800	150	666	983	881	664	946
Above 800	22	879	1058	97	863	989

Arranged according to milk production

Milk yield	Actual weight			Estimated weight		
	Number of cows	Average milk yield	Average weight	Number of cows	Average milk yield	Average weight
lb.		lb.	lb.		lb.	lb.
Under 5000	28	4,658	793	245	4,712	756
5,000–10,000	1022	7,550	867	8274	7,516	844
10,000–15,000	298	11,697	950	1913	11,464	934
Above 15,000	23	16,548	1082	88	16,082	985

Arranged according to butter-fat percentage

Butter-fat	Actual weight			Estimated weight		
	Number of cows	Average butter-fat	Average weight	Number of cows	Average butter-fat	Average weight
%		%	lb.		%	lb.
Under 4	1	3.87	1200	6	3.71	992
4 –4.9	246	4.64	887	1850	4.65	869
4.9–6.1	979	5.44	885	7587	5.46	857
6.1–7.0	143	6.33	903	1048	6.36	859
Above 7.0	2	7.09	935	29	7.16	907

between these cows would be for weight 8050 lb., height at withers 4968 lb., depth at withers 6526 lb., heart girth 5481 lb., paunch girth 6355 lb., width at hips 5731 lb., body length 5922 lb., rump length

4617 lb. The differences between the butter-fat percentages of the smallest and the largest cows are so small as to be of no significance, 0.3 per cent. being the largest difference.

These comparisons make it evident that weight is more important than the other measurements in determining the milk which the cow will secrete. This view finds confirmation in another extensive body of data which has been collected in the earlier volumes of the Registry of Merit. These include 365-day records of 1371 cows whose weights were known and 10,547 cows whose weights were estimated. No other measurements were taken. The two groups, actual and estimated weight, are analysed separately to allow comparison. In Table III the average productions and weights of these cows have been arranged in a similar manner to those in Table II.

Table III supports the conclusion that butter-fat and milk production are closely related to the weight of the cow, an increase in weight bringing about an increase in production. On the other hand, weight has no significant relation to the butter-fat percentage of the milk. It thus becomes clear that the reason why the butter-fat yield and weight are related is because of their joint relation to the milk yield. The equations showing these relations are of interest both for this discussion and for that to follow. These equations are:

Where Actual Weight Is Given

Milk yield = 1634 + 7.83 weight.
Butter-fat yield = 107 + 0.40 weight.
Butter-fat % = 5.24 + 0.00017 weight.

Where the Weight Is Estimated

Milk yield = 530 + 8.95 weight.
Butter-fat yield = 50 + 0.454 weight.
Butter-fat % = 5.59 − 0.00021 weight.

These sets of equations are in agreement in principle and in fair agreement as to actual arithmetical results. Taken together they show that an increase in a cow's weight of 100 lb. increases her milk yield 850 lb., increases her butter-fat production 45 lb., and has no effect on the butter-fat percentage contained in the milk. The data on which these equations are based are seen in Table IV.

The diagram of Fig. 2 shows that weight is the most important single element in indicating the cow's milk yield or butter-fat production. The scale used to convert this influence of weight on production is the correlation scale running from 0 to 1. On this scale weight has a relation of 0.51 to milk yield and 0.51 to butter-fat yield. The effect on the butter-fat percentage is practically nil −0.02. For the earlier Registry of Merit data the relation between the weight of the cow and her milk yield is 0.47 for those actually weighed and 0.47 for those with estimated weights. The butter-fat yields were related to the weights by 0.48 (actual weight) and 0.46 (estimated weight). For the butter-fat percentages the relations were 0.04 (actual weight) and −0.05

TABLE IV

Average, Variation in, and Relation between Weight, and Production of Jersey Registry of Merit Cows, 365-Day Records. 1371 Cows with Actual Weights, 10,547 with Estimated Weights

	Actual weight given				Estimated weight given			
	Average	Standard deviation	Correlation with		Average	Standard deviation	Correlation with	
			Age*	Weight			Age*	Weight
Age*	4 73 yrs.	2 48 yrs	—	0 376	4 41 yrs.	2 39 yrs.	—	0 498
Weight	888 lb.	121 lb.	0 376	—	860 lb.	113 lb.	0 498	—
Milk yield	8540 lb.	2454 lb.	0 501	0 468	8240 lb.	2173 lb.	0 452	0 466
Butter-fat %	5 39	0 559	−0 164	0 045	5 41	0 492	−0 105	−0 049
Butter-fat	456 lb.	121 lb.	0.478	0 480	442 lb.	111 lb.	0 438	0 463

* All correlations of age with the other variables are in error so far as measuring the true relation between them is concerned since the regression lines are skew curves.

(estimated weight). The conclusions drawn from these two sets of data are identical.

Milk yields and butter-fat productions in relation to the weights of the cows are of interest in the light of Gaines' (5, 6) study of the economy of production. Basing his study on the energy value of the milk produced, of the food consumed, and on Haecker's (15) data on the energy requirement for maintenance of live weight, he arrives at the formula

$$\text{C.E.} = 52.6 \frac{\text{F.C.M.}}{\text{F.C.M.} + 8.847w},$$

as a measure of the cow's efficiency in milk production. F.C.M. (fat-corrected milk) equals 0.4 milk yield + 15 butter-fat, and w equals

weight. This formula applied to McDowell's (18) data on milk yields of dairy herd improvement cows showed that the cows which weighed least produced their milk at a less energy cost than those cows which were heavier, to this extent contradicting current thought that in dollars the milk costs less per hundredweight for the heavier cow than for the lighter cow. The records of the Registry of Merit cows here presented are for all ages. The cows are fed considerably above the amount any feeding standard would allow. Allowing these inaccuracies it is still of interest to apply this formula to these Registry of Merit figures. From the equations on p. 522, assuming cows to weigh 600, 800, 1000, and 1200 lb., the average milk yields and butter-fat productions are found to be 7152 lb. of milk and 392 lb. of butter-fat; 9046 and 494; 10,940 and 595; 12,834 and 697 lb. respectively. These values lead to F.C.M.'s of 8736, 11,020, 13,306, and 15,591 lb. for each weight class. The C.E.'s are 32.7, 32.0, 31.6, and 31.3[1]. There is a decline in the efficiency coefficient from the 600 lb. cow to the 1200 lb. cow, but this decline is slight compared with that observed by Gaines where the C.E.'s for the same weight classes were 28.0, 26.6, 24.7, and 23.0 on cows ranging in age from 5 to 9 years. But it is to be remembered that the actual food intake (and consequent waste) was greater in these cows than that called for by the ordinary standards[2]. It is, however, of interest to note that the milk produced by both groups, when excessively fed, has ironed out the difference between the weight groups. This suggests that possibly the cows of larger weights could

[1] The numerical value of the C.E.'s is influenced by the fact that these records are 365 days in length, there being no dry period. The values are consequently greater than those observed in herd-test records as these herd-test records include a dry period of 6 weeks or so in each year's record.

[2] This difficulty may be overcome in part as follows. The effect of the heavy feeding would be to make the ratio of digestible nutrients for lactation less than 0.327 F.C.M. as determined from Haecker's data by Gaines. How much less is doubtful, but to show the effect of this decrease it may be assumed that the digestible nutrients for lactation equalled 0.5 F.C.M. Substituting this value the formula becomes

$$\text{C.E.} = 34.4\frac{\text{F.C.M.}}{\text{F.C.M.} + 5.79w}.$$

Cows would now have their new C.E.'s depending on their weight as follows: 600 lb. C.E. 24.6; 800, 24.2; 1000, 24.0; and 1200, 23.8. The observed differences are proportionately even less than those cited above.

be profitably fed a somewhat increased ration over those weighing less rather than that the cows are necessarily less efficient because of their greater weight. Gaines' comparison of the feeding results on Danish cattle shows a like effect—the Red Danish weighing 1021 lb. had a C.E. of 23.8, the cross-bred weighing 913 lb. had a C.E. of 23.8, and the Jersey weighing 796 had a C.E. of 23.9 (6). The discussion of the C.E.'s of mature cows will be presented later in the paper.

The conclusions to which these data on the Jersey breed lead can be extended to a breed as different as the Holstein-Friesian. The Holstein-Friesian Herd books record the weight, height at withers, heart girth, rump length, width at hips, 7-day milk yield, and butter-fat percentage in a much smaller body of data—385 cows. The comparison of these measurements with the 7-day milk yields shows that the weight is the most important single factor in the cow's production. The other measurements are of about equal importance when compared with each other but of less significance to milk yield than weight. No measurement has any influence on the butter-fat percentage found in the milk.

Since similar conclusions may be drawn from data on two breeds as divergent in size as Jerseys and Holstein-Friesians, these conclusions appear to apply generally to dairy cattle. Factors of body size making for body mass tend materially to affect milk and butter-fat production. The most influential of these body measurements is weight: the larger the cow the larger her probable production. The other body measurements are essentially skeletal measurements having little regard to the amount of fleshing. These measurements are of about equal importance to the cow's production. They do not, however, have an importance equal to the cow's weight in indicating her production. These measurements, or elements of type, have little or no relation to the percentage of butter-fat the cow is able to secrete in her milk. The measurements are sufficiently distributed over the cow's body to allow the conclusion that any comparison of cows for a given part would be related to the cow's milk production in such a manner that the larger the part the larger the production. This fact holds when the measurements are considered individually and without regard to the cow's age. The question of the influence of the cow's age on these relations may now be discussed.

Mature-Form Type and Its Influence on Production

The influence of age on the relation between conformation and milk secretion may be eliminated by comparing only cows of the same age, or by determining the relation of age to each variable and then correcting that variable for age effect. The latter method was selected as the most satisfactory. The age of 8 years 3 months was chosen as that to which to make these adjustments. This is the age at which growth is very nearly stationary, having been essentially completed.

TABLE V

Average Size, Variation in Size, and Correlation between Measurements of Conformation and Production in 840 Jersey Registry of Merit Cattle, Old Requirement. All Records Are Those of the Mature Equivalent at 8 Years 3 Months[1]

	Average	Standard deviation	Correlation with		
			Milk yield	Butter-fat %	Butter-fat
Age	—	—	−0.074	−0.042	−0.073
Weight	937 lb.	115 lb.	0.272	0.080	0.298
Height at withers	47.8 in.	1.72 in.	0.167	0.134	0.221
Depth at withers	27.7 in.	1.54 in.	0.128	0.107	0.165
Heart girth	71.9 in.	3.46 in.	0.043	0.150	0.104
Paunch girth	88.6 in.	5.02 in.	0.118	0.101	0.159
Width at hips	20.8 in.	1.50 in.	0.106	0.077	0.131
Body length	37.9 in.	2.41 in.	0.134	0.077	0.164
Rump length	21.0 in.	1.34 in.	0.113	0.113	0.151
Milk yield	10,719 lb.	2588 lb.	—	−0.210	0.901
Butter-fat %	5.40	0.535	−0.210	—	0.208
Butter-fat	570 lb.	146 lb.	0.901	0.208	—

[1] The data and methods by which these records were brought to uniform mature-form measurement and production are given in the papers to follow.

The cow is at what may be regarded as her mature-form type. Growth curves for each of the eight conformational measurements and for milk yield, butter-fat production, and butter-fat percentage were determined on the 5000 records which were available. From these age curves factors were determined which would place measurements made at different ages on the same mature-form basis.

The relative value of these points of conformation to the milk production, butter-fat production, or butter-fat percentage, may best be

shown by a bar diagram similar to that used in Fig. 2. The length of the bars in Fig. 3 is based on the size of the correlation coefficients in Table V between type and production on a mature-form basis.

The comparison of Fig. 2 with Fig. 3 shows the striking effect of age on the correlation of conformation and production. Making age

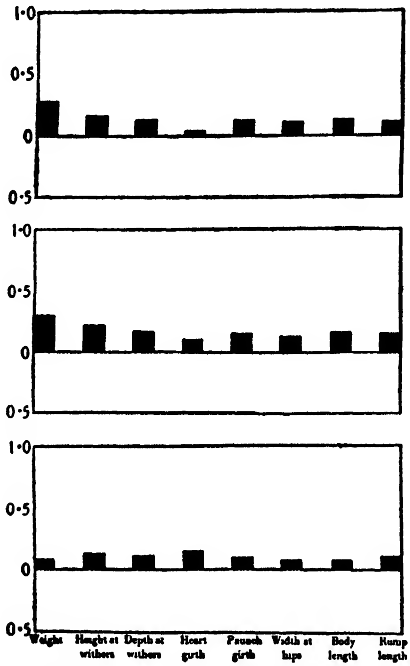

FIG. 3. Relation between conformation and production of Jersey Registry of Merit cows at mature form (age-corrected for conformation and production). The height of the bars represents the value of the parts of conformation as a means of indicating production. Weight of the cow is clearly the most important element of conformation for showing the cow's probable mature-form production.

uniform has had the effect of reducing materially the observed relation of type and production.

Milk and butter-fat production still have a greater relation to type than does the butter-fat percentage. Correcting for age has, however, revealed a slight correlation between type and butter-fat percentage. Weight is still more important than skeletal measurements in indicating the probable performance of a cow in milk yield or butter-

fat production. This is apparently due to the fact that weight includes a measure of not only the skeletal size but also the degree of fleshing. In other words, the greater the weight the greater the animal's probable production. The skeletal measurements have about equal value in indicating what the animal will produce for milk or butter-fat. Heart

TABLE VI

Average Production and Size Measurements for Jersey Cattle

A. Arranged according to the butter-fat production of these cows. Age-corrected records

Butter-fat	Number	Butter-fat	Weight	Height at withers	Depth at withers	Heart girth	Paunch girth	Width at hips	Body length	Rump length
lb.		lb.	lb.	in.	in.	in.	in.	in.	in.	in.
Under 400	85	371.3	898.0	47.3	27.5	71.9	88.0	20.7	37.4	20.8
400–600	459	503.0	919.0	47.6	27.6	71.6	88.0	20.7	37.7	20.9
600–800	234	684.0	962.0	47.9	27.8	72.1	89.2	21.0	38.3	21.2
Above 800	62	905.3	1025.0	48.8	28.5	73.1	91.0	21.3	38.9	21.4

B. Arranged according to the milk production of these cows. Age-corrected records

Milk yield	Number	Milk yield	Weight	Height at withers	Depth at withers	Heart girth	Paunch girth	Width at hips	Body length	Rump length
lb.		lb.	lb.	in.	in.	in.	in.	in.	in.	in.
Under 10,000	373	8,470	909.0	47.5	27.5	71.8	88.2	20.7	37.6	20.9
10,000–15,000	411	11,960	953.0	47.9	27.8	71.8	88.7	20.9	38.1	21.1
Above 15,000	56	16,605	1002.5	48.4	28.3	72.7	90.5	21.2	38.5	21.3

C. Arranged according to the butter-fat percentage of these cows. Age-corrected records

Butter-fat	Number	Butter-fat	Weight	Height at withers	Depth at withers	Heart girth	Paunch girth	Width at hips	Body length	Rump length
%		%	lb.	in.	in.	in.	in.	in.	in.	in.
Under 4	2	3.90	950.0	48.0	27.5	71.0	85.0	20.5	37.0	20.5
4–5	204	4.73	927.0	47.5	27.5	71.2	87.9	20.6	37.7	20.7
5–6	530	5.48	937.5	47.8	27.7	71.9	88.7	20.8	37.8	21.0
Above 6	104	6.31	950.5	48.2	28.2	72.8	89.3	21.1	38.8	21.3

girth apparently has the lowest relation to production when taken by itself. When placed in its proper perspective, however, with the weight and other measurements taken into consideration, heart girth is found to have more significance, for as will be shown in the succeeding section, a relatively small heart girth, or a wedge-shaped form, is the conformation which favours milk production.

The data may be arranged to show the average age corrected measurements for each item of type for different grades of butter-fat production, milk yield, and butter-fat percentage. These results are shown in Table VI.

The results of Table VI show a fairly steady increase in the size of the animal as the butter-fat or milk yield is increased. In other words, the larger the butter-fat yield or milk yield, the larger the cow is found to be. This increase is most marked for the weight of the cow. The larger the milk yield or butter-fat yield the greater the weight of the cow.

The butter-fat percentage shows a more uniform behaviour than it did for the case of cows at all ages. This is partially due to the fact that age has a negative relation to the percentage of butter-fat which a cow is able to secrete in her milk. That is, the older the cow, the less the average proportion of butter-fat in her milk. The correction of the butter-fat percentage records to a mature-form basis has consequently eliminated this variable.

The data may be arranged to give the average milk yield, butter-fat production or butter-fat percentage for given weights or body measurements of the mature cows. The equations are

Butter-fat = 215.1 + 0.379 weight.
Butter-fat = 18.8 height at withers − 329.8.
Butter-fat = 134.5 + 15.7 depth at withers.
Butter-fat = 254.7 + 4.4 heart girth.
Butter-fat = 160.1 + 4.6 paunch girth.
Butter-fat = 304.5 + 12.8 width at hips.
Butter-fat = 192.3 + 9.9 body length.
Butter-fat = 22.42 + 16.5 rump length.

Milk yield = 5001 + 6.11 weight.
Milk yield = 252.1 height at withers − 1321.
Milk yield = 4670 + 215.0 depth at withers.
Milk yield = 8382 + 32.5 heart girth.
Milk yield = 5321 + 60.9 paunch girth.
Milk yield = 6915 + 182.8 width at hips.
Milk yield = 5264 + 144.0 body length.
Milk yield = 6140 + 218.3 rump length.

Butter-fat % = 5.05 + 0.00037 weight.
Butter-fat % = 3.40 + 0.042 height at withers.
Butter-fat % = 4.37 + 0.037 depth at withers.
Butter-fat % = 3.37 + 0.023 heart girth.
Butter-fat % = 4.45 + 0.011 paunch girth.
Butter-fat % = 4.83 + 0.028 width at hips.
Butter-fat % = 4.76 + 0.017 body length.
Butter-fat % = 4.45 + 0.045 rump length.

These equations are used in the same manner as those given earlier for cows of all ages. Perhaps the most significant comparison is that indicating what the probable production of the largest cow at mature form would be as contrasted with the probable production of the smallest cow at mature form. The largest Jersey cow at mature form is 1300 lb. in weight, 53 in. height at withers, 32 in. depth at withers, 81 in. heart girth, 100 in. paunch girth, 24 in. width at hips, 45 in. body length, and 25 in. rump length. The smallest cow is 600 lb. in weight, 43 in. height at withers, 22 in. depth at withers, 61 in. heart girth, 74 in. paunch girth, 17 in. width at hips, 29 in. body length, and 18 in. rump length. The cows largest in weight produce 265 lb. more butter-fat than the smallest cows, the largest in height at withers 188 lb. more, the largest in depth at withers 157 lb. more, heart girth 88 lb. more, paunch girth 120 lb. more, width at hips 89 lb. more, body length 159 lb. more, rump length 115 lb. more. For milk yield the increased productions would be: weight 4273 lb., height at withers 2521 lb., depth at withers 2150 lb., heart girth 650 lb., paunch girth 1584 lb., width at hips 1280 lb., body length 2304 lb., and rump length 1528 lb. The butter-fat test for the larger cows would be expected to be slightly more than that for the smaller cows, as follows: for weight 0.3 per cent., height at withers 0.4 per cent., depth at withers 0.4 per cent., heart girth 0.56 per cent., paunch girth 0.3 per cent., width at hips 0.2 per cent., body length 0.3 per cent., and rump length 0.3 per cent.

The data which have been presented show that conformation in general has a small but rather consistent relation to the milk-producing capacity of the cow. The degree of this correlation is about equal to that found in inheritance studies for the relation of the milk yield of half-sisters. Separating the different elements of conformation we find that of the eight measurements weight has the largest numerical

relation to the milk production. This fact is further borne out by the correlations derived from another set of Jersey data published in the early Jersey Registry of Merit volumes. These correlations for weight of the cow and her milk production for a constant age (age being eliminated by grouping the data in age classes) are shown in Table VII.

TABLE VII

Relation between Production and Weight for Constant Age

Age groups years	Milk yield	Butter-fat yield	Butter-fat %	Number of cows
		Estimated weights		
Under 2	0.15	0.16	−0.10	750
2 -2½	0.10	0.05	−0.18	2215
2½-3	0.14	0.10	−0.15	971
3 -3½	0.21	0.24	−0.05	994
3½-4	0.18	0.21	−0.10	750
4 -5	0.14	0.15	−0.13	1331
5 -6	0.20	0.22	−0.16	1125
Above 6	0.28	0.22	−0.27	2411
Average.......	0.18	0.16	−0.17	—
		Actual weights		
Under 2	0.24	0.38	0.23	57
2 -2½	0.29	0.27	0.07	270
2½-3	0.25	0.40	0.26	114
3 -3½	0.05	0.08	0.05	116
3½-4	0.24	0.31	0.15	93
4 -5	0.38	0.40	0.09	186
5 -6	0.29	0.34	0.17	166
Above 6	0.31	0.34	0.03	379
Average.......	0.28	0.32	0.10	—

The correlations of Table VII in the two divisions estimated and actual weights are consistent in showing a slight but significant effect of weight on milk and butter-fat yield. The numerical values for the estimated weight are generally somewhat less than those for the actual weight. The correlation of weight and butter-fat percentage of the estimated and actual groups is of opposite sign but generally small in amount. These data are thus confirmatory to those of Table V.

On p. 527 we discussed the question of efficiency of production in the light of Gaines' work, the concept being applied to the relation of production to weight where age was allowed to vary. The question may now be discussed for the relation of weight to production for a constant age. Gaines has studied the cow's efficiency of production within the age range 5–9 years, the assumptions behind the formula being those previously indicated. The data here differ from those of Gaines in that the cows are in all probability fed considerably beyond their needs. In view of the push to get cows into the Registry of Merit and to make as good a record as possible it seems probable that each weight class is equally extravagantly fed. It is useful to examine the milk yields which are thus attained in relation to what they should be on the basis of equal efficiency for each weight class of cows. The data calculated by Gaines' formula,

$$\text{C.E.} = 52.6 \frac{\text{F.C.M.}}{\text{F.C.M.} + 8.847w},$$

lead to the following values of the coefficient of efficiency for the different weight classes: 600 lb. 29.0, 800 lb. 30.5, 1000 lb. 31.6, and 1200 lb. 32.5. In calculating these results the expected average milk yields and butter-fat productions are obtained from the mature-form equations of p. 532. On pure-bred Jerseys under herd-test management Gaines shows that the efficiency coefficients for the different weight groups are 600 lb. 28.0, 800 lb. 26.6, 1000 lb. 24.7, and 1200 lb. 23.0. Comparison of these figures with those given above shows that the benefit of the excess care, feeding, and selection of the Registry of Merit cows has distinctly improved the production of the larger cows over that of the smaller cows, bringing both sets of animals into essentially the same level of production. This change suggests that the larger cows need more understanding management than do the smaller cows when under herd conditions.

The data thus far obtained indicate that there is a small, almost too small to be practically very useful, relation between the cow's conformation and her production. There is no significant relation between the cow's type and the butter-fat percentage of her milk. So far as these measurements cover the subject, weight is the most determinative item. Second to weight is the cow's heart girth. The other measurements have but slight influence on production.

In its early history the American Jersey Cattle Club collected scores on Registry of Merit animals. The analysis of the relation of the scores to production indicates the same general conclusions expressed above where actual measurements were available. The score card data show (7, 8, 9) that the wedge-shape with large, deep paunch is an important item in indicating what a cow will do in the way of milk production. The score card data further bring out the point that the size and condition of the udder and the characteristics of the milk veins are significant elements of conformation for indicating the milk-producing capacity of the cow[1]. This conclusion is further substantiated by studies on actual udder size in relation to milk secretion (4, 13, 14, 19, 20, 21). The work is supplemented by that of Aldrich and Dana (1), which reaches the conclusion that the condition of the milk veins is somewhat indicative of the cow's milk yield.

In view of these facts the following general conclusions may be drawn. From a production standpoint the significant items in mature form conformation or type are: the size of the cow, the size and characteristics of the udder and milk veins, and the wedge-shaped form characteristic of dairy cattle.

The conformational items even when taken together are rather inferior indicators of production, having a value about equal to that of the record of a half-sister in showing what the cow's record will be. The problem may now be examined from the standpoint of conformation taken as a whole.

Conformation as a Whole and Its Relation to Milk-Producing Capacity

In the preceding sections an evaluation has been made of the relation of the different parts of conformation to production. This information, while essential to any analysis of the problem, does not entirely solve the relationship between type or conformation and production. The idea of conformation carries with it the idea that the cow viewed as a whole—instead of just as parts—is related to the milk yield she is able to produce. This ideal can only be reached when each individual measurement is considered in relation not only to milk yield but also

[1] Unfortunately the measurement data forming the basis of this paper give no information on udder size or milk veins.

in its proper setting with regard to the other measurements. For this purpose the eight measurements of conformation of the Jersey Registry of Merit cows may be considered together by contrasting their total effect with the production of the cow. To accomplish this end it is necessary to measure the effect on the rate of milk secretion of each of

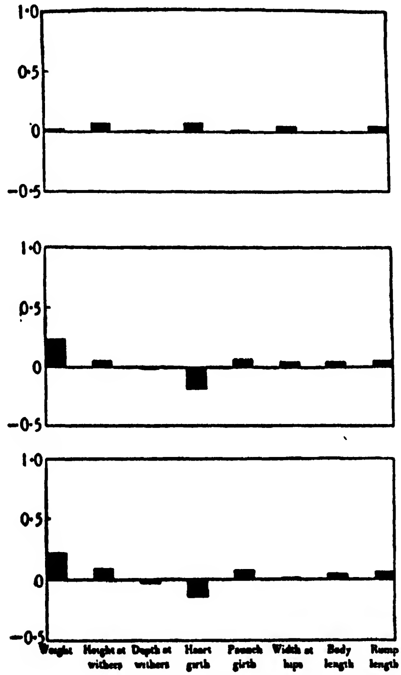

FIG. 4. Relation between conformation and milk secretion. The effect of the given part on milk secretion as measured is distinct from any influence of the other parts which might be correlated with it. When the bars extend above the zero line an increase of the given part results in an increase of the milk secreted. When they are below the line a decrease in the size of the part results in an increase of the milk secreted. The relative influence of the given part on milk secretion is measured by the scale at the left graduated from zero, no influence, to one, complete control.

the parts of conformation as distinct from any relation they may have with each other. Or, stated differently, each part of conformation must be measured and related to milk production in a group of cows having the variation induced by the other parts eliminated. Thus the

true influence of the particular element of conformation as related to milk secretion may be determined. The resulting constants, the partial correlation coefficients, are numerical measures of the relation between given items of conformation and milk secretion; these are the constants which the judges who criticise the score card have been demanding.

Fig. 4 shows the influence of the eight different measurements of conformation as entirely separate from each other. The height of the bars represents the relative influence of the given part of conformation as it is correlated with production when any influence the other parts

TABLE VIII

Partial Correlation Coefficients

First variable	Second variable, other measurements constant*		
	Milk yield	Butter-fat %	Butter-fat
Age	—	—	—
Weight	0.233	0.020	0.219
Height at withers	0.053	0.068	0.087
Depth at withers	−0.010	0.010	−0.017
Heart girth	−0.181	0.073	−0.145
Paunch girth	0.063	0.014	0.071
Width at hips	0.037	0.049	0.014
Body length	0.036	0.000	0.041
Rump length	0.053	0.044	0.065

* The height of the bars in Fig. 4 represents these partial correlation coefficients.

might have is eliminated. The bars which extend above the zero line show that an increase in the size of the given part results in an increase in milk yield, butter-fat production, or butter-fat percentage as the case may be. When the bars extend below the zero line a decrease in the size of the part results in an increase in milk secretion. The relative influence of the given factor on milk secretion is measured by the scale at the left graduated from zero, no influence, to one, complete control. These data are found tabulated in Table VIII.

Three facts are shown by Fig. 4. First, the weight of the cow for her age is the most important point of the eight studied when related to milk yield. The larger the cow for her age the larger her milk yield.

The numerical value of this relation is so small as to be difficult to handle in practice.

The cow's heart girth is the second significant point. The cow should have a relatively small heart girth for her size and age. The measurement of the cow's depth at the heart girth enables us to determine what this fact means. A section through the cow at the region of the heart is an ellipsoid something like that shown in Fig. 5. The outer line of the ellipsoid is the measurement of the heart girth. The line A, from withers to chest floor, is the measurement for the depth at withers. It is clear that broadly speaking the heart girth is determined by the depth at the withers, line A, and the breadth across the chest, line B. Examination of Fig. 4 shows that the depth has no relation to milk production when considered in and of itself, but that the cow should have a relatively small heart girth to be a typically high milk producer. Consequently to get this relatively small heart girth, the length of the line B must be relatively short—the breadth across the chest and the filling in the withers must be relatively small. The cow should show the wedge-shaped form when viewed from the front. It is of interest to pause a moment and speculate as to what this sharpness over the withers means in the cow's physiological economy. If we revert to the time-honoured custom of comparing the extreme beef type of cow with the cow giving large quantities of milk it is noted that it is in this region that the beef cow puts on flesh readily. The beef cow in contrast to the dairy cow is noted for its lack of a point at the withers; it is broad and blocky in this region. The dairy cow, on the other hand, has but a little covering of flesh in this region. Her food does not seem to make flesh; it seems rather to be used by the udder and converted into milk. There is a specific difference in the animal's internal physiology which may be observed in her external appearance. The results of the analysis of these Jersey data furnish the first clear-cut proof that this reasoning may be applied to well-bred cows secreting large amounts of milk.

The third point brought out by a study of Fig. 4 is the uniform relative unimportance of the other six points of conformation to the cow's milk yield. It is clear that the cow's weight since it measures the skeletal growth and degree of fleshing, and the wedge-shaped form when viewed from the front, are points influencing milk secretion in

dairy cows. These points are brought about by the pattern of the cow's growth. That a specific type of growth pattern is related to the mammary glands' activity in these so-called normal cows has a significance comparable to the conclusion that growth and the secretions of the pituitary are related to pattern of growth.

While we have uniformly spoken of conformation as it is related to milk yield, the facts are just as true for the butter-fat yield of the cow—due to the close association between quantity of milk flow and quantity of butter-fat contained within it and the small correlation between the percentage of butter-fat and quantity of milk flow.

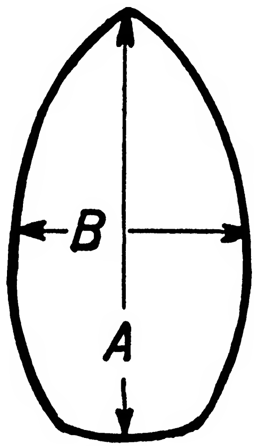

FIG. 5.

The negative side of the evidence is of equal interest. The fact that the measurements of depth at the withers, paunch girth, and body length show no marked association with the amount of milk secreted casts doubt on the significance of the side wedge as related to milk yield. It is in fact doubtful if this wedge has any relation to the milk production of high-class dairy cows.

The fact that the length of the rump fails to show any important relation to the amount of milk secreted causes us to believe that the argument for the length of rump indicating the length of the udder attachment and therefore the size of the udder and its milk yield has no basis in reality. This feeling of distrust is further borne out by the fact that the width at the hips, another point supposed to measure

the size of the udder attachment and therefore the milk flow, also shows no important relation to the cow's milk yield.

The width at the hips and the body length are functions of the other body wedge supposed to be indicative of milk secretion. This is the wedge as it is observed in looking down upon the cow. As neither of these measurements has any important association with milk production it would follow that this wedge has but little significance in pointing out the high producing cow.

The negative conclusions hold for the butter-fat yields to essentially the same degree as for milk production.

The case for conformation as related to the butter-fat percentage in the milk is entirely negative. Fig. 4 shows clearly that none of the eight measurements of conformation has any intrinsic relation to the butter-fat percentage. This conclusion is in accord with that in the literature on a more extended list of conformational measurements. The data before us as they are today point to the conclusion that the cow's ancestry or an actual test on her milk are the only reliable means available for predicting the butter-fat percentage within the milk of a normal cow.

The data for milk and butter-fat yield may be collected in equation form where each of the measurements is given its proper weight in predicting milk yield or butter-fat production. These equations are as follows:

$$\begin{aligned}\text{Milk yield} &= 4743 + 6.69\,\text{wt.} + 102\,\text{H.W.} - 17\,\text{D.W.} - 228\,\text{H.G.} \\ &\quad + 45\,\text{P.G.} + 113\,\text{W.H.} + 48\,\text{B.L.} + 171\,\text{R.L.} \\ \text{Butter-fat yield} &= 0.352\,\text{wt.} + 9.3\,\text{H.W.} - 2.0\,\text{D.W.} - 10.2\,\text{H.G.} \\ &\quad + 2.9\,\text{P.G.} + 2.3\,\text{W.H.} + 3.1\,\text{B.L.} + 11.7\,\text{R.L.} - 79.4\end{aligned}$$

In these equations wt. represents the weight in pounds. All other measurements are in inches. H.W. is height at withers; D.W. is depth at withers; H.G. is heart girth; P.G. is paunch girth; W.H. is width at hips; B.L. is body length; and R.L. is rump length. Granting that the ideal of conformation is the cow of high milk yield, these equations furnish a means of measuring the cow's probable milk yield from these measurements and therefore her relative approach to the ideal type. Furthermore, it gives this approach to the ideal on a numerical scale of milk production which is capable of being compared from one cow to another. The actual practical application of these results to judging may be illustrated by the measurements of two cows. The first cow

was a large-sized Jersey having the following measurements: weight 1300 lb., height at withers 53 in., depth at withers 32 in., heart girth 81 in., paunch girth 100 in., width at hips 24 in., body length 45 in., and rump length 25 in. From these measurements the probable milk yield of this cow would be $4743 + 6.69 \times 1300 + 102 \times 53 - 17 \times 32 - 228 \times 81 + 45 \times 100 + 113 \times 24 + 48 \times 45 + 171 \times 25 =$ 13,481 lb. of milk.

The second cow chosen was of small size for a mature cow. Her measurements were: weight 600 lb., height at withers 43 in., depth at withers 22 in., heart girth 61 in., paunch girth 74 in., width at hips 17 in., body length 29 in., and rump length 18 in. The probable milk yield as determined from these measurements would be $6.69 \times 600 + 102 \times 43 - 17 \times 22 - 228 \times 61 + 45 \times 74 + 113 \times 17 + 48 \times 29 + 171 \times 18 =$ 8582 lb. of milk. The relative values of the two cows' conformations are thus in the relation of 13,481 to 8582. On the basis of such a measurement of conformation the larger cow is obviously the better in milk yield. This does not mean that she necessarily produces milk more economically. The discussion of pp. 527 and 535 indicates rather that the smaller cow may be equally economical of food stuffs or take less actual energy per pound of milk than the large cow.

It should not be supposed that these measurements account for all the variation in milk yield or butter-fat production in dairy cattle. The eight measurements, in point of fact, contain within them only two of the four elements known to be important to milk production, weight and heart girth. The other important elements, size and quality of the udder and milk veins, when properly taken into consideration, would account for much more of the variation in the milk yield. The predicted milk yield and the actual milk yield will agree only when a large group of cows is taken into consideration and the results averaged. For individual cases the variation of the actual milk yield from the predicted milk yield will still be large. In fact the eight measurements of conformation account for only 6 per cent. of the total variation in milk yield or butter-fat production in Jersey Registry of Merit cows. Or, put in actual terms, for a predicted 10,000 lb. milk yield as derived from these eight measurements there would be only an even chance that the actual milk yield would lie between 7561 and 12,433 lb. Thus it is obvious that a cow's potential milk yield is

nearly invisible in her external make-up. This is even more true for the butter-fat percentage of the milk. To mention but one important item, the cow's inheritance for milk secretion is far more significant than her conformation.

The Ideal Type of Cow

The facts just presented may be collected and coordinated with the other analysed data as found in the literature in discussing the form taken by the average cow secreting a large quantity of milk. It is to be noted that practically all the critical material is found in measurements of Jersey or Holstein-Friesian cattle of good milking capacity—these remarks therefore bear particularly on these breeds although they are believed to be of general significance. These studies have been directed almost wholly toward the relation of the cow's conformation and her milk production.

Five or six concrete facts are to be gleaned from these studies. The age of the cow is always related to her milk production and to her conformation in such a manner that as age advances the milk production and the size increase up to mature form. In fact the age of the cow is as fundamentally important to milk production as the total effect of the eight points which are here analysed (see also (2, 10, 22)). This joint increase of milk yield and size with age creates a partially spurious correlation between milk yield and conformation. This spurious correlation may best be eliminated by judging only within age classes or, if it is necessary to compare animals of different ages, to correct the measurements of both conformation and milk production to a standard age as was done for these studies. In the further discussion it is assumed that all records are age-corrected so that age no longer is a factor in the relation of type to production.

The weights of dairy cattle are related to their milk secretion in such a way that a relatively large weight means a relatively large milk flow. The food intake necessary to maintain this larger size may however partly or wholly overcome the advantage of weight. It seems to depend in part on the measure utilised in determining the economy of production.

At the same time these cows should show relatively little fleshing at the withers giving a wedge-shaped appearance when viewed from the

front. These three facts find confirmatory evidence in a study of measurements of cows within the Holstein-Friesian breed (11), and of score-card records of Jersey Registry of Merit cows (7).

Besides the measurements taken for this study there are several others of importance. The first and foremost is the size and quality of the udder. The Jersey score-card data (7) show that when measured by score, the size and quality of the udder are the leading qualifications of the heavy milking cow. The shape of the udder and the arrangement of the teats are of but minor significance as far as milk yield is concerned. This fact finds its strongest support in studies of actual udder weights related to the milk yields of these udders (4, 13, 14, 19). In these studies cows whose daily milk production and stage of lactation were known, were killed, the udders dissected off and weighed, and their composition determined. These results show that there is a close association between the udder tissue present and the amount of milk secreted, thus establishing the great importance of the size of the mammary gland to milk production.

The blood passing through the udder as it brings the basic materials from which milk is made is important to milk secretion. As an index of this blood supply it has been customary to use the appearance and size of the mammary veins, or milk veins. Aldrich and Dana (1) measured and compared the size of these veins with the milk flow for about 600 cows. Their analysis shows that there is a relation between the size of these veins and the milk secreted by the udder sufficient to warrant their careful consideration in dairy cattle judging. The score-card data of the American Jersey Cattle Club likewise support this view (7).

On the negative side it is shown that where the age, weight, and the wedge-shaped form are taken care of the rest of the body measurements such as height or depth at withers, paunch girth, width at hips, body length, or rump length are of little importance. It is further clear that only one of the body wedges need be given consideration—that viewing the cow from the front, the side wedge and back wedge having little significance.

Before leaving a subject which has been given so much prominence in the writings on animal husbandry it seems desirable to make a comparison between the different methods by which it is possible to estimate the probable future production of the cow. These methods

are the cow's conformation for her age, a previous milk record of any length, and her heredity for milk secretion (the record of production shown by her ancestors). A rough idea of the significance of these possible methods in indicating the outcome of a future lactation may be obtained from the squared correlation coefficients between the given items and milk secretion. These data for Jersey Registry of Merit cows are indicated by the height of the bars in the accompanying diagram (Fig. 6).

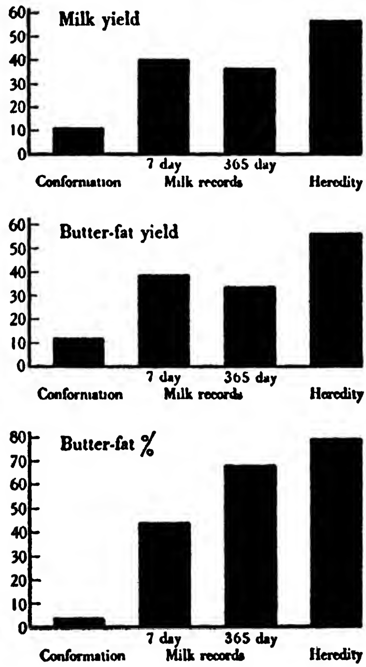

FIG. 6. Comparative value of conformation, 7-day or 365-day records, and heredity, for predicting milk secretion in Jersey cattle.

The data of Fig. 6 are for the eight conformational points discussed in this paper; the relation of a 7-day record to a 365-day record when the 7-day record is a part of the 365-day record; a 365-day record related to a subsequent 365-day record; and the effect of heredity as determined from the dam and grand dams. The chart shows that conformation on the whole gives but little real information on the cow's subsequent production as compared to the other methods which are, theoretically at least, available.

SUMMARY

This paper presents an analysis of the relation borne by eight body measurements and age to the cow's milk-secreting ability. The analysis is first presented from the viewpoint of conformation as related to milk secretion without regard to age. The data are then utilised to determine the relation of conformation to milk secretion when age is considered. In the last section we have attempted to consider conformation as a whole and to relate this to milk secretion. Defining the ideal as quantity production of milk or butter-fat the following points of conformation are important. First, the cow should be of better than average weight for her breed and age; second, she should be of good wedge-shaped form particularly in the region of the shoulders; third, her milk veins should be of good size; and fourth, her udder should be of good size and quality. Time has brought about the inclusion in the animal husbandry text-books of many other points asserted on *a priori* grounds to be of significance in determining the cow's milk-secreting capacity. The effect of several of these points on milk secretion has been analysed in these data, the results showing them to be without significance.

A few words of caution: if the measure of worth is to be the relation of energy intake of the food the animal consumes to the energy found in the milk then the effect of weight on quantity of milk produced seems to be offset by the extra energy necessary to maintain this weight. It is also shown that the cow's conformation is on the whole a rather inferior means of predicting future milk-producing capacity. This does not mean that the writer is in favour of neglecting the information it can give for it has one real advantage over the other method: it shows the present status of the cow's physical health and whether the mammary gland is physiologically normal.

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THE CONFORMATION OF THE PARENTS AS RELATED TO THE MILK SECRETION OF THE DAUGHTERS, JERSEY REGISTRY OF MERIT

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Conformation has an interest not only because of its supposed relation to the milk which the cow will be likely to give, but also because of the assertion that the animal whose conformation is desirable will transmit high milk yield or butter-fat percentage to its offspring. This belief is commonly maintained for the bulls, since it is argued that a bull of pleasing conformation, a winner in the show ring, is the bull to beget offspring which will be winners at the pail. These assertions were more common 20 years ago, when dairymen did not bother to keep records, than they are to-day. However, they are common enough to-day to warrant investigation of the facts.

There were 208 Jersey cows with Registry of Merit records by sires whose measurements were taken. One hundred and twenty-two cows came from measured dams. While these data are not large in amount they furnish the only available information in existence on this important problem. The measurements of conformation include estimated weight, height at withers, depth at withers, heart girth, paunch girth, width at hips, body length, and rump length. The skeletal points delimiting each measurement are shown in a photograph in a former paper on p. 517. All the records have been placed on the same age-corrected basis so that they are strictly comparable.

Table I shows the average daughter's production for sires of different conformation as indicated by their body measurements.

The study of Table I reveals no outstanding relation between the

TABLE I

Relation between Sire's Conformation and Daughter's Production

Sire's weight	Daughter's average			Sire's height at withers	Daughter's average			Sire's depth at withers	Daughter's average			Sire's heart girth	Daughter's average		
	Milk yield	Butter-fat	Butter-fat		Milk yield	Butter-fat	Butter-fat		Milk yield	Butter-fat	Butter-fat		Milk yield	Butter-fat	Butter-fat
lb.	lb.	%	lb.	in.	lb.	%	lb.	in.	lb.	%	lb.	in.	lb.	%	lb.
1075	8,000	5.8	450	48.5	7,000	5.8	400	28.5	7,900	5.82	455	73	8,800	5.56	478
1125	9,450	5.3	490	49.5	11,050	5.5	605	29.5	10,950	5.40	588	75	9,600	5.76	538
1175	10,375	5.4	555	50.5	9,450	5.4	493	30.5	11,300	5.02	553	77	8,800	5.62	485
1225	9,750	5.9	550	51.5	9,950	5.2	505	31.5	11,800	5.40	635	79	10,600	5.16	537
1275	9,250	5.7	510	52.5	10,800	5.3	568	32.5	10,500	5.36	550	81	11,150	5.40	595
1325	10,450	5.0	510	53.5	13,650	5.5	743	33.5	9,450	5.58	510	83	11,750	5.36	623
1375	10,200	5.3	515	54.5	9,350	5.5	525	34.5	9,550	5.60	520	85	11,350	5.50	613
1425	12,500	5.5	705	55.5	11,750	5.2	600	35.5	12,900	5.04	638	87	—	—	—
1475	12,250	5.5	655	56.5	9,900	6.0	605					89	9,400	5.84	533
1525	10,475	5.6	585												
1575	12,075	5.6	670												
1625	8,875	5.3	458												
1675	11,750	5.2	600												

Sire's paunch girth	Daughter's average			Sire's width at hips	Daughter's average			Sire's body length	Daughter's average			Sire's rump length	Daughter's average		
	Milk yield	Butter-fat	Butter-fat		Milk yield	Butter-fat	Butter-fat		Milk yield	Butter-fat	Butter-fat		Milk yield	Butter-fat	Butter-fat
in.	lb.	%	lb.	in.	lb.	%	lb.	in.	lb.	%	lb.	in.	lb.	%	lb.
83	10,850	5.22	552.5	17.5	7,750	5.90	462.5	33	7,750	5.90	462.5	19.5	7,750	5.90	462.5
85	—	—	—	18.5	—	—	—	35	—	—	—	20.5	11,100	5.04	542.5
87	9,250	5.86	532.5	19.5	11,750	5.42	630.0	37	—	—	—	21.5	11,100	5.40	595.0
89	8,250	5.76	462.5	20.5	10,200	5.38	540.0	39	10,700	5.24	540.0	22.5	11,300	5.28	585.0
91	10,800	4.96	532.5	21.5	10,800	5.22	547.5	41	9,600	5.58	522.5	23.5	10,550	5.54	577.5
93	12,650	5.52	695.0	22.5	11,850	5.24	617.5	43	9,300	5.38	487.5	24.5	9,800	5.26	497.5
95	9,350	5.52	510.0	23.5	8,800	5.32	460.0	45	12,600	5.34	670.0	25.5	9,800	5.60	535.0
97	9,000	5.46	485.0	24.5	9,300	5.72	517.5								
99	11,250	5.58	615.0												
101	15,000	4.60	687.5												
103	—	—	—												
105	9,800	5.60	535.0												
107	8,100	5.10	405.0												

TABLE II

Relation between Dam's Conformation and Daughter's Production

Dam's weight	Daughter's average			Dam's height at withers	Daughter's average			Dam's depth at withers	Daughter's average			Dam's heart girth	Daughter's average		
	Milk yield	Butter-fat	Butter-fat		Milk yield	Butter-fat	Butter-fat		Milk yield	Butter-fat	Butter-fat		Milk yield	Butter-fat	Butter-fat
lb.	lb.	%	lb.	in.	lb	%	lb.	in.	lb.	%	lb.	in.	lb.	%	lb.
675	11,750	5.7	688	43.5	11,250	6.15	713	23.5	5,750	6.3	363	65	8,400	5.4	445
725	5,750	6.3	363	44.5	11,050	5.4	590	24.5	11,250	4.7	513	67	9,900	4.9	473
775	9,950	5.4	530	45.5	10,450	5.3	540	25.5	9,650	5.3	523	69	11,200	5.6	620
825	10,200	5.4	528	46.5	11,150	5.3	585	26.5	10,550	5.4	553	71	12,150	5.5	663
875	9,550	5.4	505	47.5	10,000	5.3	515	27.5	11,350	5.3	600	73	10,300	5.3	525
925	11,300	5.3	593	48.5	10,800	5.4	575	28.5	11,200	5.6	615	75	9,800	5.4	520
975	10,700	5.4	563	49.5	11,300	5.5	625	29.5	11,000	5.3	570	77	9,950	5.4	523
1025	12,800	5.5	705	50.5	11,300	5.5	618	30.5	9,250	5.4	480	79	—	—	—
1075	10,950	5.4	580	51.5	9,400	5.4	483	31.5	12,250	5.7	688	81	15,000	5.2	775
1125	12,350	5.4	653												
1175	10,250	5.6	563												

Dam's paunch girth	Daughter's average			Dam's width at hips	Daughter's average			Dam's body length	Daughter's average			Dam's rump length	Daughter's average		
	Milk yield	Butter-fat	Butter-fat		Milk yield	Butter-fat	Butter-fat		Milk yield	Butter-fat	Butter-fat		Milk yield	Butter-fat	Butter-fat
in.	lb.	%	lb.	in.	lb.	%	lb.	in.	lb.	%	lb.	in.	lb.	%	lb.
77	13,250	4.3	588	17.5	9,250	5.4	483	29.5	12,250	5.6	688	18.5	10,050	5.1	513
79	8,900	5.6	475	18.5	10,750	5.2	538	32.5	13,000	5.9	738	19.5	11,200	5.4	595
81	8,150	5.4	438	19.5	11,000	5.4	593	33.5	8,000	5.1	375	20.5	11,400	5.4	610
83	10,850	5.4	583	20.5	11,300	5.5	620	34.5	10,850	5.3	558	21.5	10,800	5.4	580
85	12,250	5.6	685	21.5	10,550	5.4	550	35.5	9,000	5.5	495	22.5	9,950	5.4	523
87	10,700	5.4	560	22.5	10,200	5.4	528	36.5	9,950	5.1	463	23.5	10,650	5.5	563
89	10,850	5.5	585	23.5	11,300	5.5	598	37.5	9,900	5.3	518	24.5	7,750	5.6	425
91	10,450	5.3	550					38.5	9,750	5.4	508				
93	11,450	5.3	593					39.5	9,700	4.9	528				
95	8,650	5.7	475					40.5	10,350	5.0	503				
97	11,150	4.8	525					41.5	10,950	5.7	595				
99	—	—	—					42.5	12,100	5.2	630				
101	15,000	5.2	775					43.5	7,250	5.9	413				

conformational measurements of the sire and the production of the daughters. The weight of the sire seems to have a slight importance in indicating what the daughter's probable production will be in milk and butter-fat yield. No measurement of the sire appears to be of any particular importance to the butter-fat percentage. From these

TABLE III

Constants of Variation and Correlation between Sire's Conformation and the Milk Production of His Daughters

	Average size	Standard deviation	Correlation coefficients between sire's type and daughters' production*		
			Milk yield	Butter-fat %	Butter-fat
Sire's:					
Weight	1381 lb.	140 lb.	0.20	0.06	0.22
Height at withers	52.7 in.	1.48 in.	0.11	0.10	0.15
Depth at withers	32.2 in.	1.25 in.	−0.11	0.08	−0.10
Heart girth	80.5 in.	2.69 in.	0.17	0.05	0.18
Paunch girth	93.5 in.	5.48 in.	−0.07	0.17	−0.01
Width at hips	21.4 in.	1.61 in.	−0.13	0.06	−0.12
Body length	42.6 in.	1.92 in.	0.23†	0.06	0.24
Rump length	22.7 in.	2.32 in.	−0.07	0.00	−0.05
Daughters':					
Milk yield	10,796 lb.	2822 lb.	—	—	—
Butter-fat %	5.38	0.503	—	—	—
Butter-fat	571 lb.	159 lb.	—	—	—

* Probable error 0.05.

† The body-length data were inadvertently grouped in small groups, all but three observations coming in the last four groups. The first three of these classes showed a negative relation between increase in body length and increase in milk yield. The last class makes the observed correlation. In view of the whole trend however this correlation appears to be without significance.

facts the tentative conclusion may be drawn that the sire should be of good weight to produce daughters whose production of milk will be above average. This relation, however, is not a close one so that it cannot be emphasized.

The average productions of the daughters for the conformational measurements of the dams are shown in Table II.

Like Table I for the sire, the data of Table II are irregular, showing but a small relation between the conformation of the dam and the production of the daughter. Here again weight of the dam is the most important element of conformation in indicating what the probable production of the daughter will be. The other body measurements show practically no relation to the daughter's production.

TABLE IV

Correlation between Dam's Conformation and Her Daughters' Production

	Average size	Standard deviation	Correlation coefficients between dam's type and daughters' production*		
			Milk yield	Butter-fat %	Butter-fat
Dam's:					
Weight	928 lb.	110 lb.	0.26	0.01	0.25
Height at withers	47.9 in.	1.86 in.	0.02	0.11	0.05
Depth at withers	28.0 in.	1.42 in.	0.03	0.02	0.03
Heart girth	71.7 in.	3.29 in.	0.03	−0.04	0.00
Paunch girth	88.5 in.	4.46 in.	0.06	0.08	0.01
Width at hips	20.7 in.	1.43 in.	0.00	0.03	−0.01
Body length	37.9 in.	2.65 in.	0.01	0.01	−0.01
Rump length	21.1 in.	1.36 in.	−0.13	0.10	−0.11
Daughters':					
Milk yield	10,824 lb.	2891 lb.	—	—	—
Butter-fat %	5.41	0.574	—	—	—
Butter-fat	575 lb.	168 lb.	—	—	—

* Probable error 0.06.

The constants of variation and correlation between the sire's and dam's conformations and the daughters' milk yield, butter-fat production, and butter-fat percentage are seen in Tables III and IV.

SUMMARY

The above data on the sire's conformation and on the dam's conformation in relation to their daughter's production of milk are concordant in showing that with the possible exception of weight, there is no correlation of practical significance between the body type of the

sire or the dam and the productive capacity of the daughter in milk, butter-fat, or butter-fat percentage.

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PRODUCTION OF FATAL INFESTATIONS IN RABBITS WITH TRICHOSTRONGYLUS CALCARATUS (NEMATODA)*

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While the nematode genus Trichostrongylus contains more than a dozen species which are known to parasitize at least that many hosts, chiefly herbivores but including man, and while pathogenicity is freely attributed to the superfamily Strongyloidea, of which this genus is a member, the author knows of no experimental work clearly indicting any trichostrongyle as a pathogen. In the course of experiments which demonstrated that *Trichostrongylus calcaratus* Ransom, 1911 (1) would cause the development of an acquired resistance in the domestic rabbit (*Oryctolagus cuniculus*) (2), evidence accumulated that this nematode was pathogenic and able to kill rabbits when present in large numbers. Premonitory symptoms, mentioned in the order of their appearance, include diarrhea, loss of appetite, loss of weight, general unthriftiness, and progressive emaciation. Such symptomatology, while of the type frequently ascribed to "worms," in the case of *T. calcaratus* is almost pathognomonic. Since at least three trichostrongyle species are reported as parasites of man, and several other species are of common occurrence in domesticated animals, attention may well be directed to determining whether the presence of trichostrongylids in large numbers is more strictly associated with such symptoms.

The writer is not cognizant of any previous work demonstrating experimentally the pathogenicity of a rabbit helminth, although lesions in rabbit stomachs parasitized by *Obeliscoides cuniculi* have

* The writer wishes to acknowledge the helpful advice of Dr. Norman R. Stoll in planning and starting this research, as well as in the preparation of the material for publication.

been reported by Chandler (3) and Alicata (4). Schwartz (5) points out that "the extent of the injuries produced by worms in rabbits is not definitely known, inasmuch as the worm parasites of these animals have not been extensively studied," but on the basis of reputed pathogenic effects of closely related worms in other animals he ascribes the familiar symptomatology of "weakness, emaciation, and anemia" to roundworm infestation of the rabbit's stomach and small intestine.

Methods

In this article data are presented on experimental infections in 14 rabbits carefully followed as to clinical manifestations and weights and by egg counts to determine the course of the parasitic infection. The procedures utilized were in general those previously described (2). Rabbits were secured in litters when weaned and placed on screens in separate cages which were sterilized each week. All the rabbits of each litter received the same adequate ration. Frequent fecal examination over a period of at least 3 weeks indicated that the rabbits were not parasitized by worms and were losing their naturally acquired coccidial infections. The animals were infected orally by pipette with infective *T. calcaratus* larvae recently isolated from 10-day room-temperature cultures of dung from rabbits harboring pure infections of this helminth. At autopsy the gross pathology was noted, and each portion of the gastro-intestinal tract was stripped repeatedly and material thus obtained carefully screened for worms through screens of 20, 40, and 60 meshes to the inch. Although *T. calcaratus* is an inconspicuous nematode only 5–7 mm. long and of thread-like diameter, by this method at least 95 per cent of the worms actually present in the rabbits are believed to have been recovered. In the graphs each point indicates a separate observation, indicating in the case of the flotation or dilution egg counts a carefully established average on a fecal specimen collected over a period of 24 hours or more. Open circles on the base line indicate that no eggs were found. For brevity the letter "R" is used for "Rabbit" in the graphs and text, and to avoid repetition the comparative data concerning each animal's infection are not given in detail in the text but are summarized in table 1. A preliminary report of this research was presented

before the American Society of Parasitologists at Atlantic City in December, 1932 (6).

Experiments and Results

Repeated infection experiment. This experiment was originally part of a test for the occurrence of the self-cure phenomenon (i.e., "the

TABLE 1

Summary of Data on Three Types of T. calcaratus Infection

Rabbit no.	No. of infections	Total larvae given	Dosage ratio†	Age in weeks when 1st infected	Maximum egg count		Final egg count		Total *T. calcaratus* recovered at autopsy	Weeks from infection to autopsy	Weeks to 1st effect on weight	Weight lost (per cent)	Average loss of wt. (Kg. per week)	Per cent of control's wt. at end of exp.
					EPG	EPD	EPG	EPD						
					(a) Fatal infestations									
1	8	56,000	25	21	16,300	327,000	3,700	44,000	1,642	8	5	35	0 22	61
6	9	83,000	24	104±	3,200	111,000	2,500	9,000	1,002	9	1	42	0.18	
10	1	18,000	7	27	11,500	200,000	3,400	34,000	1,344	72/7	4	40	0.22	60
14	1	10,000	7	12			2,100		1,946	71/7	3	35	0.13	57
17	1	17,000	13	15	13,600	504,000	9,000	504,000	4,616	95/7	3	29	0.15	61
					(b) Infections which had some effect on weight									
5	10	126,000	36	52±	5,000	373,000	800	30,000	1,124		5	6	0.11	
7	16	159,000	63	104±	1,600	116,000	0	0	0		3	21	0.10	
15	1	9,000	9	9	14,600	351,000	0	0	1		3			77
					(c) Infections which had no effect on weight									
3	10	125,000	36	52±	3,300	275,000	33	1,700	91					
8	1	260	0.1	16	500	45,000								100
9	1	2,500	1.2	18	940	108,000								85
11	1	2,000	1.4	12			48	860	18					102
12	1	5,000	3.7	12			1,200	54,000	1,014					94
16	1	15,000*	16*	10	4,500	144,000	0	0	4					103

* Larvae were 2–4 weeks old.
† Thousands of larvae per kilogram of rabbit.
EPG = eggs per gram.
EPD = eggs per day.

acquirement of a resistance which causes the expulsion of the worms present" (7)) using a more rapid rate of infection than in the author's previously reported experiment (2), but is of greatest interest here as

the initial demonstration of fatalities due to *T. calcaratus*. Comparison was made of the infections produced by increasing, weekly, oral doses of larvae in 5 rabbits of different ages and parasitic histories. Figure 1 shows the course of the resulting infections in terms of the number of parasite eggs passed in the feces of each rabbit and fig. 2 summarizes graphically the clinical effect of the infestations as measured by changes in the weight of the rabbits.

Rabbit 1, a comparatively young and previously uninfected rabbit, was very susceptible to infection at first, then became resistant to superimposed infection, and finally began to throw off the worms which it had acquired, as shown by the decreasing egg count, the passing of worms, and the abnormal position of the worms at autopsy. However, before self-cure could be completed the large infestation which the animal was carrying brought about its death, preceded by symptoms of chronic diarrhea, lack of appetite, and progressive emaciation. The details concerning this fatal *T. calcaratus* infection were as follows:

Rabbit 1 was first infected when 21 weeks of age and received 56,000 larvae in 8 doses. Worm eggs appeared in its feces on the 13th day, increased in number up to the 5th week, and then decreased rapidly. The falling egg count was associated with diarrhea and passage of worms, 7 worms being recovered from 33 gm. of liquid feces on the 35th day, 217 from 30 gm. on the 47th day, and 49 from 27 gm. on the 51st day. Occasional masses of soft fecal pellets were passed during the first few weeks of the infection and diarrheic feces in the 4th week. In the 6th week the rabbit began to lose weight and had a fetid odor due to fecal soiling. During the 6th and 7th week it refused to eat, daily evacuated large amounts of liquid feces containing strands of mucus, and was constantly soiled. In the 8th week it was noticeably emaciated, constantly soiled with foul smelling rancid feces, walked doubled up as if cramped, and did not eat. It continued to lose weight, and was found dead exactly 8 weeks after the first infection. It had lost 870 gm., representing 35 per cent of its weight, in less than 4 weeks, and at death weighed 61 per cent as much as its uninfected litter mate.

At autopsy the cecum and the large intestine were flatulent and no feces were present in the latter; the first foot of the small intestine contained gelatinous mucus and a few worms, the 2nd and 3rd feet a pasty mixture of mucus and food débris, and the remaining 4½ feet watery mucus and flatus. There was no evidence of hemorrhage into the lumen of the small intestine, and no gross lesions in the intestines or elsewhere, although 126 worms were recovered from the small intestine, 1007 from the cecum, and 509 from the large intestine. Total worms 1642; many from the cecum and large intestine were of small size.

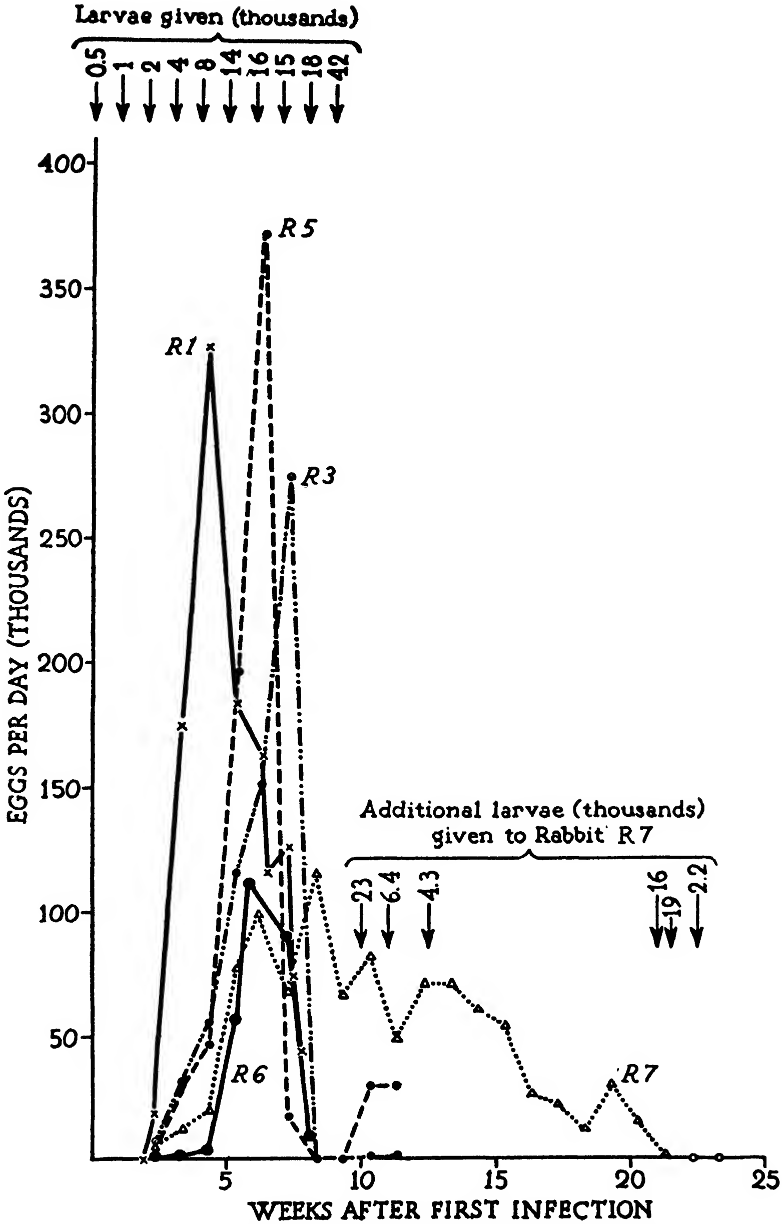

FIG. 1. Parasitic infections produced in rabbits by increasing weekly doses of *T. calcaratus* larvae. An initial susceptibility was followed by the acquirement of resistance to superimposed infection, and for 4 of the 5 rabbits by loss of infection through the "self-cure" process.

Rabbits 3 and 5 were about one year old and of unknown parasitic history, having been obtained through the kindness of Dr. John B. Nelson after they had developed precipitins following a series of injections with *Ascaris lumbricoides* (from pig) extract. The injections apparently had no effect on their susceptibility to *T. calcaratus*

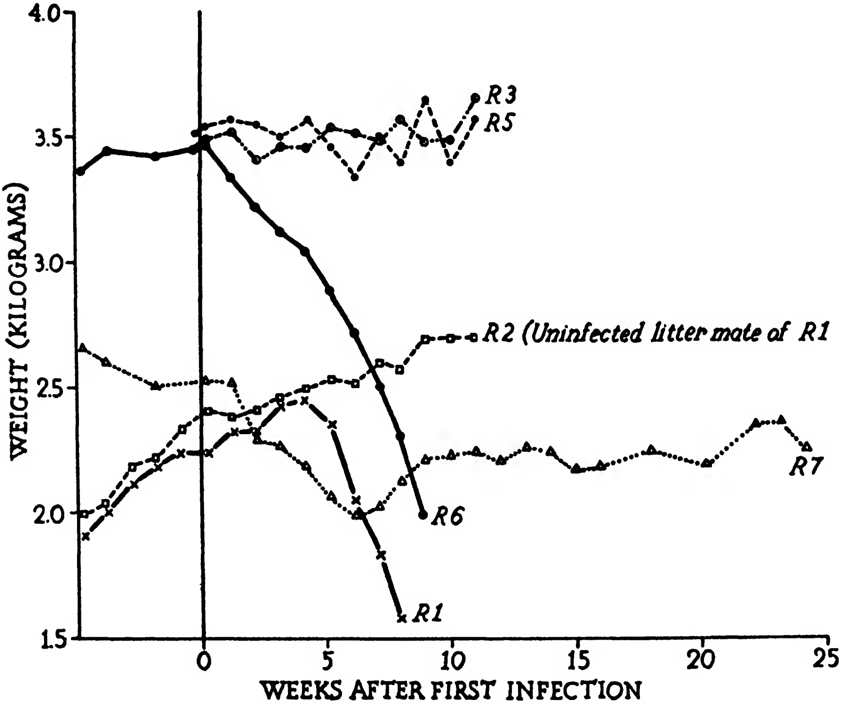

FIG. 2. Weight curves for the rabbits whose egg counts are shown in fig. 1. Marked loss of weight occurred in 2 fatal infections (R 1 and R 6) and temporary loss of weight in 2 non-fatal infections (R 7 and R 5).

infection. Both exhibited increasing egg counts following the first few doses of larvae which they received, then became resistant to superimposed infection, and finally eliminated most of the worms which had been acquired. Rabbit 5 lost weight temporarily at the peak of its infection, and following self-cure some of the largest doses of larvae evidently broke through its newly acquired resistance to establish a new infection of considerable size. Rabbit 3 did not lose weight. It proved highly resistant to the large final doses of larvae

which it received, and passed diarrheic feces frequently at the height of its infection.

Rabbit 6, an approximately two year old animal carrying a low grade *T. calcaratus* infection of prolonged duration, died following exposure to the increasing doses of larvae. Its record was as follows:

The first three doses of larvae produced only a small infection in R 6 but the 4th and 5th doses were followed by increases to a peak of infection in the 7th week and by sharp decreases in the 8th and 9th weeks when the moribund rabbit was killed. Rabbit 6 had often appeared soiled about the anal region previous to receiving its series of increasing infections but this condition became more marked following infection. In the first week after infection its anus was observed to be dilated, and this condition became more marked throughout the experiment so that masses of fecal pellets always protruded from the animal's rectum. Small amounts of liquid feces were passed in the 2nd and 3rd weeks, and masses of soft fecal pellets from the 4th week on. The rabbit began to lose weight in the 1st week and its weight curve continued to drop throughout the experiment. It became ill in the 7th week and a nervous system involvement, which twisted the head low and far around to the side, appeared in the 8th week, together with loss of appetite. Being prostrated and having both hind legs paralyzed, it was killed at 9 weeks after infection. At autopsy the anal region was extremely soiled and the anus was dilated. Externally R 6 was considerably emaciated, but internally there was still considerable fat present, especially about the kidneys. No gross lesions were observed except in the small intestine which was thick-walled in the first few inches, but very thin at the 25th and 49th inches where irregularly streaked with opaque white membranous patches firmly adherent to the mucosa. It yielded 9 small and 65 minute worms. The cecum was flatulent and contained 229 small worms. The large intestine contained 699 small worms and soft fecal pellets embedded in mucus. Total worms recovered 1002, all of small size and 93 per cent displaced downward from their usual location in the gut. The effect of this animal's chronic anal dilation and the terminal paralysis on the course of its infection is uncertain. They appear to have been of a secondary and contributory nature since neither has been observed in other fatal infestations.

Rabbit 7. This animal was approximately two years old. One and a half years previously, as "Rabbit 17" in an outdoor reinfection experiment reported by Stoll (8), it had shown "a clear indication of the development of a resistance resulting in a precipitate loss of worms." When infected it was not passing any worm eggs in its feces but was slowly losing weight, evidently because of a bad ear infection with mange mites. The increasing doses of larvae produced

egg counts which increased with the first few doses and then gradually decreased to a negative in the 23rd week, indicating the acquirement of a marked resistance to superimposed infection but failure to exhibit a self-cure crisis. Weight was lost during the height of the infection but was regained when the rabbit became resistant to further infection.

General discussion of reinfection experiment. This experiment offers interesting comparisons and contrasts with an earlier reinfection experiment (2) in which a much slower rate of reinfection was used. In that experiment each of 6 young and previously uninfected rabbits exhibited at first an infection which increased with the increasing doses of larvae, then became resistant to superimposed infection, and finally largely eliminated its worms through the occurrence of the self-cure phenomenon. In the present experiment in which 5 rabbits of various ages and parasitic histories were repeatedly infected with increasing numbers of larvae, each rabbit accepted an increasing amount of infection, each became resistant to superimposed infection, and 4 of the 5 apparently made an attempt to throw off their parasitic burden, although 2 died in the process.

Stoll (8) found no loss of weight in rabbits exposed to natural reinfection in an outdoor pen, and in the author's earlier experiment in which infections of large size became established the "weight curves were not significantly different from that of the uninfected control." In the present experiment 2 rabbits showed a similar absence of clinical effect, but there was also a clear-cut case of rapid loss of weight resulting fatally in a young animal (R 1), two cases of temporary loss of weight during the height of an infection (R 5 and R 7), and a case of extreme loss of weight and death in an old rabbit (R 6). Here, as in the earlier experiment, there is evidence that the acquired resistance resulting from reinfection with *T. calcaratus* is not absolute but, as is probably true in most types of resistance developing against pathogenic agents, can be broken through if the number of organisms used for infection is large enough. Although there was some variation in the rate at which the different animals accepted infection, all appeared relatively susceptible and therefore lacking in any protective resistance due to age, previous infection, or injections of non-specific worm substance.

First Experiment on Single Infections

An experiment to determine the effect of the size of a single larval dose on the course of the resulting infection and the weight of the host

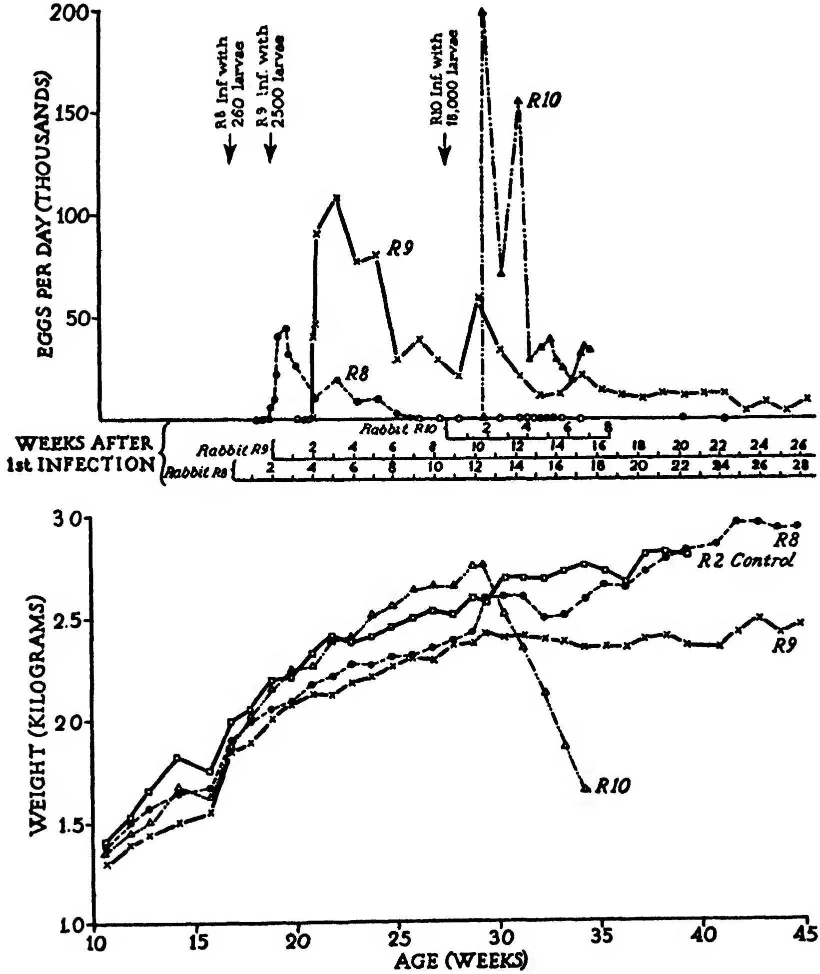

FIG. 3. Egg count and weight curves for litter mate rabbits given single infections of different sizes. Two infections had no apparent effect on weight, whereas the largest one resulted in rapid loss of weight and death.

involved 4 litter mates, 3 of which received single infections, and one kept uninfected as a control.

Figure 3 shows that the small infection which resulted from the 260 larvae fed to R 8 had no apparent effect upon this animal's weight, and it seems improbable that the divergence of R 9's weight curve from that of the control was due to parasitic infection, since numerous other infections of 2500 larvae such as R 9 received have never caused apparent retardation of growth or loss of weight, and R 9 was the least heavy member of the litter even before it was infected. The third rabbit's record will be given in detail as an example of a fatal infection resulting from a single large dose of larvae.

Rabbit 10. This animal was infected with 18,000 larvae at 27 weeks of age. Eggs appeared in its feces the 12th day and the count reached a maximum of 200,000 eggs per day on the 13th day. In the 3rd and 4th weeks the count dropped to a new level of infection where it remained with slight variations until the animal's death. In the 4th week this rabbit's weight curve, which had shown the most rapid rise of the litter, dropped sharply and continued to fall until the animal had lost 1.11 kilos, or 40 per cent of its weight, in less than 5 weeks, and at death weighed but 60 per cent of the control. Whereas R 10 passed only formed fecal pellets before infection, masses of soft fecal pellets and a small amount of liquid feces began to appear in the 2nd and 3rd weeks. Towards the end of the latter week the animal stopped eating its grain and its fecal output dropped sharply. Lack of appetite continued in the 4th and 5th weeks and feces of fetid odor and pasty consistency were passed in addition to masses of soft pellets and very small formed pellets. The rabbit still moved about actively and did not appear sick. In the 6th week it apparently ate no food, passed only a few small fecal pellets and a limited quantity of liquid feces, and was soiled about the anus. The rabbit became extremely emaciated, appeared unsteady, and walked doubled up as if with "cramps." It finally became moribund and was killed 7$\frac{4}{7}$ weeks after infection.

Post mortem the rabbit was found greatly emaciated, but the tissues did not appear anemic and exhibited no gross lesions. The first 2 feet of the small intestine contained a small amount of watery mucus and 244 worms; the 3rd and 4th feet much greenish liquid and 118 worms; the 5th to 9th feet much fluid and 435 worms. From the cecum, which was flatulent, 141 worms were recovered. The large intestine contained much gas, a small amount of feces, and 406 worms.

Rabbit 10's data present a clear case of anorexia, diarrhea, and progressive emaciation, resulting from a single large dose of larvae. The failure of the egg count to remain high during the final weeks of the infestation, the abnormal presence of 41 per cent of the worms below the small intestine, and the relatively low final egg count compared

with the large number of parasites harbored, all suggest an uncompleted attempt at expulsion of the infestation. There is a striking similarity between the infections of R 10 and R 1—although one received a single large dose of larvae and the other a series of rapidly increasing doses—as to time from infection to first loss of weight, time from infection to death, rate of loss of weight, and number and abnormal position of worms at autopsy.

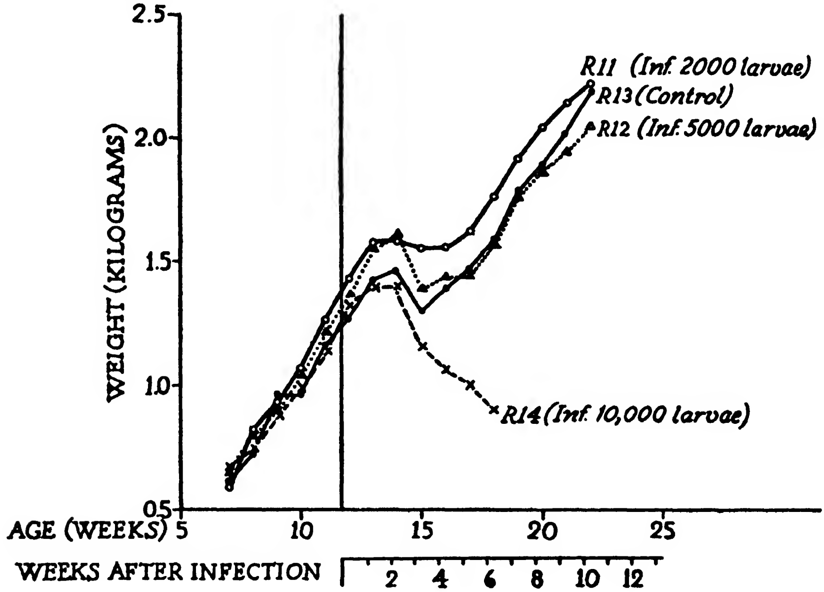

FIG. 4. Weight curves for 4 litter mates, contrasting the marked loss of weight after a fatal dose of 10,000 larvae with the absence of weight loss after infections of 2000 and 5000 larvae.

Second experiment on single infections of different sizes. Having found that a single oral infection of 18,000 larvae could be fatal, an attempt was made to produce fatalities by smaller doses of larvae in younger rabbits. Four litter mates, which were later found to be already carrying natural worm infections of small size, were placed in a single cage and three infected with the doses indicated in fig. 4. The weight curves illustrate the loss of weight which was caused by the single infection of 10,000 larvae as contrasted with the absence of any significant loss of weight following infections of 2000 and 5000

larvae. The data concerning this experiment include the following points of especial interest:

At the time of infection all four rabbits were gaining weight quite uniformly. The retarded gain in weight in the 3rd week and the loss in the 4th week exhibited by all the rabbits was evidently due to the reduction in the ration made on the 10th day after infection. All of the animals looked somewhat thin in the 5th week, but the most heavily infected one (R 14) lost weight while the others gained, and looked thinner and smaller than the others. It appeared unthrifty, its bones projected angularly under the skin, and there was little flesh to be felt on ribs or back. Weight loss and emaciation continued in the 6th week and the animal was inactive. At this time R 13 and R 12 still appeared a little thin and were not so sleek as R 11, the most aggressive of the litter. In the 7th week these three appeared more thrifty and gained weight, but R 14 was still losing weight and appeared smaller and much thinner than the others. Its arched backbone protruded apparently unsupported by muscle and it sat hunched up in a corner of the cage. At 7½ weeks after infection R 14 was dead. At autopsy extreme emaciation was found. The muscles of the back were not palpable through the skin, and the leg muscles were small and flabby. The head was drawn and rat-like in appearance. There was no mesenteric or perirenal fat. The cecum and large intestine were flatulent, as was the first 1½ feet of the unusually thin-walled small intestine, and the animal was found to harbor 1946 worms.

Rabbits 11, 12, and 13 remained in good condition throughout the experiment and showed no lesions when killed in the 11th week. None of them showed the emaciation, muscular flabbiness, or gas-filled small intestine and cecum observed in R 14, although R 12 was carrying an infestation of 1014 worms. Rabbit 11 harbored 18 worms, and R 13, the control, 6 worms.

Third experiment on single infections. In this experiment, great care was taken to treat the four litter mates involved in an identical manner, except in respect to the time and size of their infections. As in the first experiment on single infections, the infections were given successively to demonstrate more clearly the causal relationship between heavy parasitic infection and clinical effect on the host, and the rabbits were similarly previously unparasitized by worms, although passing decreasing numbers of coccidial oöcysts. Figure 5 shows the time and size of the infections, the resulting egg counts (graphed at one-half the scale of fig. 1), and the weight curves.

This experiment pictures three different clinical types of *T. calcaratus* infection in rabbits. The first, in which there is no apparent effect of the moderate worm burden on the host's appearance, activity,

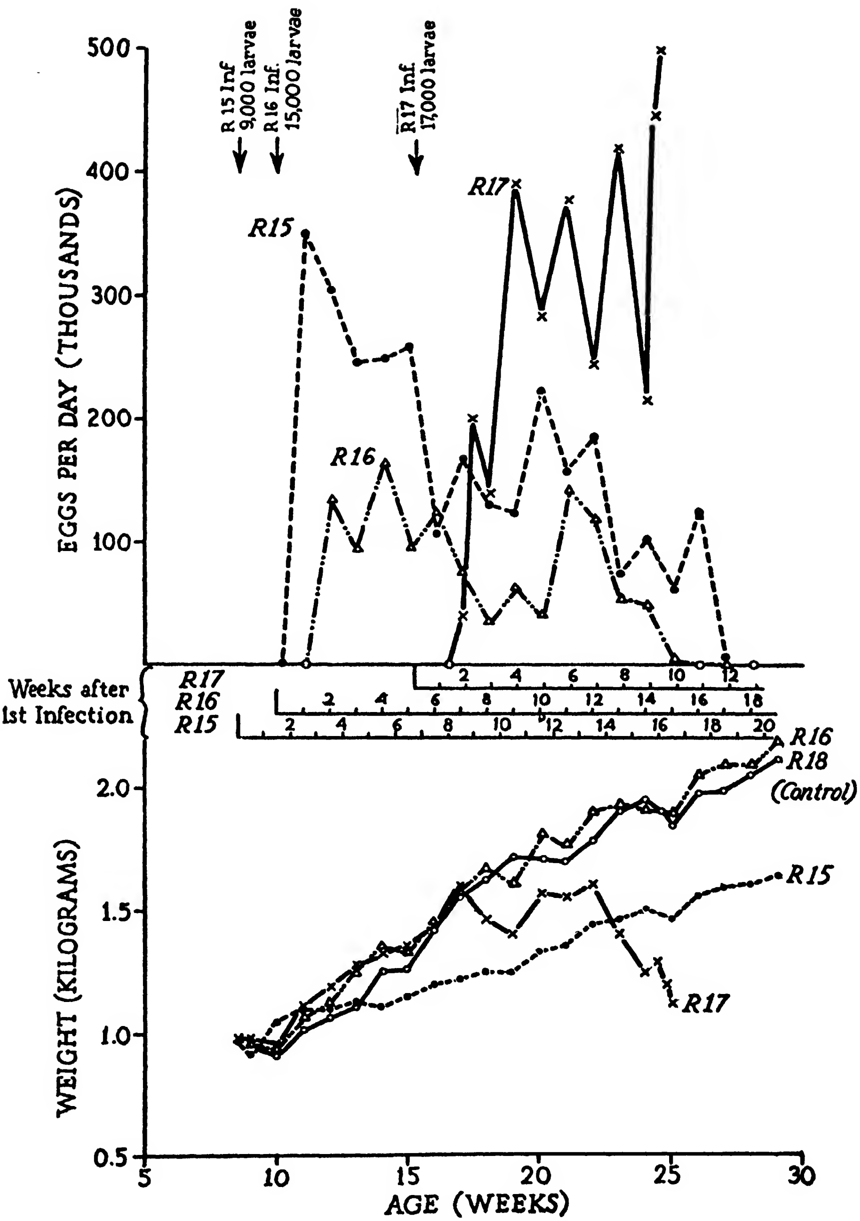

FIG. 5. Egg count and weight curves of 4 litter mate rabbits, 3 of which received a single large infection of *T. calcaratus*, showing the prolonged course of the infections and three degrees of host effect: (1) loss of weight ending in death (R 17), (2) chronically retarded gain in weight (R 15), and (3) lack of detectable effect on weight (R 16).

or growth curve, although there may be an increased tendency to passage of unformed and diarrheic feces, is typified by R 16. This animal received old larvae which were apparently of subnormal infectivity as shown by the small size of the resultant infection. The second type of infection, illustrated by R 15, is characterized by retardation of growth and weight, slight emaciation, and passage of soft and diarrheic feces. It is significant that the first indication of this retardation appeared at the same interval after infection as the first appearance of clinical symptoms in rabbits which later died as the result of their infections.

The third type of infection was produced by the large single dose of 17,000 larvae which R 17 received, and may be briefly summarized as follows:

Although formerly a normal, fat animal with a healthy appetite, R 17 began to eat poorly in the 3rd week following infection and at the end of that week looked and felt thinner than the control. It continued to eat only a portion of its food and became gradually thinner, until its backbone protruded as a rough ridge apparently unsupported by muscle. In the 8th week after infection it sat hunched in a corner of the cage with head low and back arched, was soiled with feces of a fetid odor, and had lost its former sleekness. In the 9th week it looked very sick, ate poorly, was greatly emaciated, and the anal region was soiled and protuberant. In the 10th week the rabbit was inactive; felt and looked as if it were only "skin and bones"; barely touched food; frequently passed masses of soft feces and appeared to be a very sick animal, although its rectal temperature was still normal. The rabbit finally became prostrate and moribund with a rectal temperature four degrees below normal, and was therefore killed and autopsied. Internally R 17 was found extremely emaciated, and there was a small amount of clear fluid in the body cavity. There were no gross lesions and the tissues did not appear anemic. The small intestine contained much fluid and a yellow, firmly adherent membrane in the posterior ileum. There were many worms free in the intestinal contents and attached to the mucosa and membrane, but no hemorrhage into the lumen or mucosa. The cecum was flatulent.

This fatal infection was similar in its course to the previously described fatal infestations of R 1 and R 10. It differed from them, however, in exhibiting an initial stage of slight loss and recovery of weight before the final and fatal drop in its weight curve, in the absence of chronic diarrhea, in exhibiting only partial anorexia, and in the maintenance of a persistently high egg count. Autopsy revealed that

in R 17 the majority of the worms were in their normal location, the small intestine, and the animal had been eating some food and passing formed fecal pellets. In R 1 and R 10, however, large numbers of the worms were abnormally displaced to the cecum and large intestine of animals which had been completely off diet and passing large amounts of diarrheic feces. It seems probable that the former condition was the uncomplicated result of a very large infection on a host, whereas in the latter the clinical effect of the worms was complicated by an unsuccessful attempt of the host to eliminate its parasites. The egg count curves of this final experiment show the prolonged duration of infections which result from a single large dose of larvae and are in striking contrast to the abrupt drop associated with self-cure seen in the Reinfection Experiment where the rabbits received a series of infections.

DISCUSSION

Comparative data on the three types of infections described in the present paper are given in table 1. The first group consists of 5 cases in which death followed the establishment of a large infestation. Although the rabbits in this group were of different ages and were infected by single and repeated doses, there is a striking similarity in the data. In all, the maximum and final egg counts were high; the worm burden was over a thousand worms, and the first loss of weight occurred 1 to 5 weeks after infection and was followed by death in the 8th to 10th week. The average amount of weight lost ranged from 0.13 to 0.22 kilo per week, with resultant loss of 29 to 42 per cent of the total weight, and the rabbits at the time of their death weighed only 57 to 61 per cent as much as their controls.

The second group is comprised of three cases, also infected at a heavy dosage rate, in which the weight was affected but death did not result. The loss of weight which occurred in two of these rabbits appeared at approximately the same time and progressed at the same rate as in fatal infections, but stopped when the animals became resistant to further reinfection and exhibited a decreasing egg count. The third rabbit did not lose weight but began to gain more slowly three weeks after infection and was consistently retarded in its growth thereafter.

The third group consists of six rabbits in which the infection exerted no apparent effect on the weight curve. In four of these the number of larvae fed was not more than 5000 so that the dosage ratio was less than 4000 larvae per kilogram of rabbit. The stability of weight despite large dosage ratios is apparently explained in the case of R 3 by the intervention of self-cure with resultant loss of worms, and in R 16 by poor infectivity of the old larvae which were used.

Considering only the animals in table 1 which received a single dose of larvae, it is evident that dosage ratios of 7000 or more larvae per kilogram of rabbit produced an effect on the weight curve, with the exception of R 16, whereas lesser rates of infection were without apparent effect. In 19 other single infections given to rabbits at dosage rates of 100 to 3000 larvae per kilogram of host and followed by egg counts and weekly weights, there was also no loss of weight or retardation of growth. From table 1 it would appear that for rabbits receiving a single dose of larvae the dosage ratio is a much better index of the probable effect on the host than the size of the egg count resulting from the infection. In rabbits receiving repeated infections, prediction of the effect on the host is invalidated by the possible intervention of self-cure. This was shown by the results of the first experiment of this paper and by an earlier experiment (2) in which rabbits acquired and threw off infections of considerable size when infected at dosage ratios of from 22,000 to 175,000 larvae per kilogram of rabbit.

The degree of correlation between the number of *T. calcaratus* eggs in a rabbit's feces and the number of worms present in its alimentary tract, and the factors influencing it can not be discussed here, but that there is such a correlation may be seen from even the limited data of table 1, which show that large egg counts in terms of either eggs per gram or eggs per day are associated with large numbers of worms and low counts with a small number of parasites.

The question of the manner in which heavy infestations of *T. calcaratus* exert their marasmatic effect upon rabbits awaits further investigation. Whatever the true explanation, it must account for the following composite picture of the sequence of events in a fatal infestation: A rabbit which has received a large number of larvae begins, at about the time of the maximum egg count in the third to

fourth week, to pass soft fecal pellets and liquid feces, to eat poorly or not at all, and to lose weight. These symptoms continue throughout the next 3 to 8 weeks, with the animal becoming more and more emaciated. The rabbit becomes weak, prostrated, and moribund, and finally dies 7 to 10 weeks after infection. During the terminal stages of the process the animal is usually greatly soiled with diarrheic feces of a fetid odor, and its intestines contain more or less flatus. At autopsy the rabbit exhibits an extreme emaciation, involving absence of fat from the viscera, scarcity of flesh on the bones, and general smallness and flabbiness of the muscles, but the tissues do not appear anemic. In the intestines are found 1000 or more *T. calcaratus*. The attachment of the worms to the intestinal mucosa causes no gross lesion and there is no evidence of hemorrhage into the mucosa or lumen of the gut.

SUMMARY

Laboratory rabbits were infected with *T. calcaratus* larvae in single doses of different sizes and with increasing weekly doses. The parasitic infections were followed by frequent egg counts, and the condition of the rabbits by record of weight, consistency of feces, appetite, and appearance. According to their size, single oral doses of larvae produced infections which (*a*) had no effect on the weight curve, (*b*) retarded gain in weight, or (*c*) caused loss of weight and finally death. Weekly increasing doses of larvae produced infections which (*a*) were without effect on weight, due apparently to successful intervention of the self-cure phenomenon, (*b*) caused only temporary loss of weight, evidently due to acquirement of resistance to superimposed infection with or without subsequent self-cure, or (*c*) caused gradual loss of weight ending in death, in hosts which were apparently attempting to discharge their worms when they died. In fatal infections loss of weight was associated with failing appetite, and the chronic course of infection involved progressive emaciation, frequent passage of soft fecal pellets and liquid feces, weakness, prostration, and death. Although rabbits dying of *T. calcaratus* infection always harbored more than 1000 worms, gross lesions at autopsy were usually absent except for evidences of extreme emaciation.

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THE THERMAL DEATH RATE OF TOBACCO-MOSAIC VIRUS

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INTRODUCTION

The relation between temperature and inactivation of plant viruses is useful in allowing separation of groups of viruses. Resistance of tobacco-mosaic virus to high temperature has been studied by a number of investigators. For the most part, these studies were limited to determinations of the maximum temperature to which this virus might be heated without losing its infectivity. Very little is known about the rate of inactivation of tobacco-mosaic virus at any given temperature or of the effect of different temperatures for a given length of time. The lack of a suitable and adequate method for measuring virus concentration has made it difficult and tedious to study such factors as the gradual diminution in virus concentration due to heating. Because of the recent development of the local lesion method by Holmes (5) and a modification of this method (13), it has been possible to make a quantitative study of the effect of heat on the virus of tobacco mosaic. The purpose of this paper is to present the results of this study.

REVIEW OF LITERATURE

Mayer (10) reported that the infectivity of sap of diseased plants was not perceptibly changed by continued heating at 60° C., that it was weakened by heating to 65°–70° C., and that it was completely destroyed by heating for several hours at 80° C. Iwanowsky (6) found

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that the infectious property of juice from mosaic-diseased plants was completely lost when heated to temperatures near the boiling-point. Beijerinck (2) stated that tobacco-mosaic virus was inactivated by boiling or when kept for a short time at 90° C. Raciborsky (14) reported that sap from diseased plants did not lose its infectivity when heated for five minutes at 62° C. or for one minute at 100° C., but that its infectivity was completely destroyed when heated for fifteen minutes at 100° C. Koning (9) concluded that virus diluted 1:10 with water and heated in closed tubes remained infectious when the heating process was carried on for ten minutes at 80° C., five minutes at 90° C., or five minutes at 100° C., although it was considerably weakened by heating for five minutes at 100° C. Woods (16) stated that boiling did not completely inactivate tobacco-mosaic virus in expressed juice. Allard (1) found that the thermal death point of tobacco-mosaic virus in freshly expressed juice was in the neighborhood of 90° C. for ten minutes and that the virus was quickly destroyed at temperatures above this point. He also showed that virus in dried material required much more heat for inactivation than that in solution.

Mulvania (12) found that tobacco-mosaic virus was completely inactivated when heated for ten minutes at 90° C. and was considerably weakened when heated for the same length of time at temperatures slightly below this. He observed, on the other hand, that a similar sample of virus was not completely inactivated when heated for as long as three days at 80° C. Mulvania did not observe any correlation between the water content of a virus sample and its thermal death point. McKinney (11) found that the thermal death point of virus in freshly expressed juice of mosaic-diseased plants was between 88° C. and 90° C. for ten minutes and that the thermal death point of a similar virus sample in a water dilution of 1:100 was between 82° and 84° C. for ten minutes. He concluded that the thermal inactivation of tobacco-mosaic virus is affected by the concentration of virus in the inoculum and also by the plant constituents in which the virus is contained. Brewer *et al.* (4) found that tobacco-mosaic virus (Johnson's tobacco virus 1) in freshly expressed juice of diseased tomato was completely inactivated when held at 88°–90° C. for ten minutes, or at 82°–84° C. for twenty-five minutes.

Recently, Johnson and Grant (8) have shown that tobacco-mosaic

virus is not always completely inactivated when heated for ten minutes at 90° C. but that the thermal death point is between 90° and 95° C. for ten minutes. They also studied the influence of host plant on thermal inactivation of tobacco-mosaic virus and found that the thermal death point varied as much as 5° C., depending on the host species from which the virus was obtained. Whether this variation is the result of a lower concentration of virus in some hosts, or of the effect of the host juices, was not ascertained.

Materials and Methods

The studies reported in this paper were conducted with ordinary tobacco-virus, classified by Johnson (7) as tobacco virus 1 and used by Holmes (5) and Price (13) in their studies on local lesions of tobacco mosaic. The virus samples used for heating experiments were obtained from diseased plants of *Nicotiana tabacum* L. var. Turkish, which had been infected for 30 or more days. Juice was extracted from such plants by grinding in a meat chopper and pressing by hand through one thickness of cheese-cloth.

Method of heating virus samples. In the studies where it was necessary to maintain a uniform temperature for only a short period of time (periods up to two hours), the virus samples were immersed in an electrically heated and stirred water bath, held within 0.2° C. of the desired temperature. At the end of the heat treatment, the virus samples were plunged into cold water. In such tests the virus samples were placed in containers prepared from glass tubing of 3–4 mm. bore and in 25–30 mm. lengths. At the time of testing a virus sample, one end of a tube was drawn to a fine point in a flame. Approximately two cc. (one cc. for the one-minute tests) of the sample to be tested were drawn into the tube, one end of which was then carefully sealed by heat. This treatment did not measurably decrease the infectivity of the virus sample, as was shown by tests conducted with samples immediately after preparation.

Tests were made to determine the length of time required for a virus sample to reach the temperature of the water bath in which it was placed. These tests were made by the use of a thermocouple attached to a galvanometer. The results of the tests showed that from 40 to 45 seconds were required for the temperature of the solution in the tube to reach the temperature of the bath. In 30 to 35 seconds after

immersion there was a difference of one degree in temperature of the two solutions; in 25 seconds there was a difference of four degrees in temperature. The tests were made in a water bath held at temperatures of 100° C., 90° C., and 80° C., respectively. In the heating experiments shown below, no allowance was made for the time required for the temperature of the virus solution to reach the temperature of the bath. The time periods shown in the table represent the actual time the tubes were immersed. Thus, a virus solution that was kept for one minute in a bath held at 95°C. was actually maintained at 95° C. for only 15 to 20 seconds, from 95° to 94° C. for ten seconds, and from 94° to 91° C. for ten seconds.

In experiments in which it was necessary to maintain a uniform temperature over a period of time longer than two hours, a slightly different procedure was followed. Approximately three cc. of a virus sample were placed in a tightly stoppered 1 x 10 cm. test tube. Such tubes were kept in electrically heated ovens for the duration of the test. The temperature was taken at the level of the tubes in the ovens. The oven temperature, as a rule, did not vary more than ± 1° C., but occasionally a variation of as much as 2° C. was observed. In the later experiments the tubes of virus were immersed in a paraffin bath kept inside the ovens.

Method for measuring virus concentration. The method employed by previous workers for testing for presence of virus in samples of heated juice depended upon the appearance of the systemic disease in plants of *Nicotiana tabacum* inoculated with such samples. Usually five or ten plants were used for each sample tested and the results were given in percentage of infection. The appearance of the systemic disease in this host indicates the presence of virus in the inoculum but gives little indication of the relative concentration of virus unless a large number of plants are used. The local lesion method (5, 13) for measuring virus concentration not only differentiates between presence and absence of virus but also allows a reasonably accurate estimation of virus concentration. The method was employed in the studies reported in this paper. Lesions made their appearance in about 36 hours and were usually counted in five or more days after inoculation. The test plants were grown in a greenhouse in which the temperature was not allowed to fall below 68° F.

Some of the experiments reported in this paper were conducted with

Nicotiana glutinosa L. as the test plant and the remainder with *Phaseolus vulgaris* L. var. Early Golden Cluster as the test plant. Several of the experiments with *N. glutinosa* were duplicated with *P. vulgaris*, thus allowing a comparison to be made between the two test plants. It is of interest to note that there was a close agreement between the results obtained with these two hosts.

In cases in which *Nicotiana glutinosa* was used as the test plant, five leaves of this host were inoculated with the sample tested. Plants used in these experiments were selected for uniformity of leaves at the time of making the test. In those cases in which *Phaseolus vulgaris* was used as the test plant, each virus sample was tested by inoculation of 16 primary leaves, which were almost fully expanded.

EXPERIMENTAL

Thermal death point is usually defined as the minimum temperature that results in complete inactivation after exposure for a definite period of time under specific conditions. A ten-minute period is usually taken as a standard. Similarly, thermal death time may be defined as the length of time required for complete inactivation of a virus sample when exposed to a definite temperature under specific conditions. The writer determined the thermal death point of tobacco-mosaic virus for a ten-minute exposure and for a one-minute exposure and the thermal death time for temperatures of 68°, 75°, 80°, 85°, and 90° C. The details of these experiments are given below.

Thermal Death Point: Ten-Minute Exposure

Seven different experiments were conducted in order to determine the thermal death point (ten-minute exposure) of tobacco-mosaic virus in freshly expressed juice of diseased Turkish tobacco plants. These experiments were conducted at different times of the year and included seven different virus samples. Table 1 shows the results of four experiments in which *Nicotiana glutinosa* was used as a test plant. The results of three experiments in which Early Golden Cluster beans were used as test plants are given in table 2. These results indicate that there is a slight variation in thermal death point of different samples of virus, the minimum thermal death point observed being 91° C., the maximum 93° C. On the other hand, it is possible that this seem-

ing inconsistency resulted from variation in test plants and the failure to detect minute quantities of virus in some instances rather than from any variation in virus samples. It is concluded from the results of these experiments that the thermal death point of tobacco-mosaic virus in freshly expressed juice of mosaic-diseased tobacco plants is 93° C. for ten minutes.

The results given in tables 1 and 2 also show that partial inactivation of virus occurs at temperatures well below the thermal death point and that this inactivation increases with rise in temperature. In all cases observed a temperature of 87° C. (six degrees below the thermal death point) caused a significant decrease in virus concentration.

Effect of water dilution on thermal death point: ten-minute interval. It is well known that the thermal death point of various organisms is influenced by the nature of the dispersing medium and the number of organisms in the sample. It is, therefore, of interest to know whether the thermal relations of tobacco-mosaic virus, as well as of other viruses, are influenced by such factors. It is already known that the thermal death point of tobacco-mosaic virus is appreciably lowered by dilution (11). Several experiments were undertaken in order to obtain more detailed information regarding the effect on thermal death point of dilution with water. Freshly extracted juice of mosaic-diseased plants diluted 1:20 with water was used in these experiments. Table 3 shows the results of six tests with juice diluted 1:20 with water. With three of the virus samples tested, the thermal death point was found to be 88° C. for ten minutes; with one sample, the thermal death point was 87° C.; with the remaining samples 86° C. These results show that dilution with water significantly lowers the thermal death point of tobacco-mosaic virus.

It is also of interest, in connection with these experiments, to note that, as in the case with undiluted juice, temperatures considerably below the thermal death point cause a marked decrease in virus concentration and that the degree of inactivation increases with rise in temperature.

Effect of dilution with healthy tobacco extract. Since it has been shown that the thermal death point of tobacco-mosaic virus is significantly lowered by dilution with water, it is of interest to know whether this decrease is the result of the decrease in virus concentration or of a

decrease in total solids or of both. Such a question is difficult to answer until it is possible to work with a purified virus solution. Some information regarding it has been obtained, however, by using juice of healthy tobacco plants as a diluting medium. In such a dilution, the concentration of virus can be considerably lowered without any appreciable decrease in solid content of the solution. Such a solution is not strictly comparable with undiluted juice of mosaic-diseased plants, since the presence of virus in the plant no doubt alters the properties of the juices of such a plant. It does, however, allow a rough comparison of the effect of dilution of virus and dilution of solids in the dispersing medium.

Thermal death point determinations were made with juice of mosaic-diseased plants diluted 1:20 with juice of healthy tobacco plants. The failure of control plants to produce lesions when inoculated with juice from these healthy plants proved that the juice used for dilution was free of any appreciable quantity of tobacco-mosaic virus. The results of five tests with virus in freshly extracted juice of mosaic-diseased plants diluted with healthy juice are given in table 4. In two tests, the thermal death point of virus in this dilution was found to be 91° C.; in three tests, 90° C. A comparison of these results with those presented in tables 1, 2, and 3 shows that the thermal death point of virus diluted 1:20 with juice of healthy tobacco plants is two degrees lower than that of undiluted juice and three degrees higher than that of virus in juice diluted 1:20 with water. It is, therefore, concluded that the thermal death point of tobacco-mosaic virus is influenced by the concentration of virus as well as by the nature of the dispersing medium.

An interesting phenomenon is clearly shown by comparison of tables 1, 2, 3, and 4. Although the concentration of virus in the samples used for tests shown in table 4 (juice diluted 1:20 with healthy tobacco juice) was reduced 20 times, the number of lesions produced upon leaves of *Nicotiana glutinosa* by inoculation was only slightly less than that produced by inoculation with undiluted juice. The cause of this phenomenon is not known. It may be due to the presence in healthy juice of some substance that has an effect similar to that of norit or talc, as reported by Vinson and Petre (15).

Effect of filtration on thermal death point. Since the results of previ-

TABLE 1

Effect of Exposure of Tobacco-Mosaic Virus to Various Temperatures for Ten Minutes

Temperature in degrees C.	Test No. 1	Test No. 2	Test No. 3	Test No. 4	Total	Average
86	154[a]	440	1000	528	2122	530
87	104	777	657	367	1905	476
88	138	491	516	239	1384	346
89	96	209	116	435	856	214
90	69	24	10	108	211	53
91	6	0	0	18	24	6
92	1	0	0	0	1	1
93	0	0	0	1	1	1
94	0	0	0	0	0	0
95	0	0	0	0	0	0
Unheated controls	1812	2700	2650	1236	8398	2099

[a] Figures represent numbers of lesions produced upon 5 leaves of *Nicotiana glutinosa* by inoculation with virus sample indicated.

TABLE 2

Effect of Exposure of Tobacco-Mosaic Virus to Various Temperatures for Ten Minutes

Temperature in degrees C.	Test No. 1	Test No. 2	Test No. 3	Total	Average
87	795[a]	2272	2936	6003	2001
88	759	3944	3312	8015	2672
89	123	1712	862	2697	899
90	50	928	512	1490	497
91	0	11	5	16	5
92	0	1	2	3	1
93	0	0	0	0	0
94	0	0	0	0	0
95	0	0	0	0	0
96	....	0	0	0	0
Unheated controls	3435	3488	5550	12473	4158

[a] Figures represent numbers of lesions produced upon 16 leaves of Early Golden Cluster beans by inoculation with virus sample indicated.

ous experiments showed that the thermal death point of tobacco-mosaic virus is influenced by the concentration of virus and by the concentration of solids in the dispersing medium, it appeared that

further information regarding this phenomenon might be obtained by studying the effect of filtration. Although it is well known that tobacco-mosaic virus is infectious after passage through a Berkefeld filter candle, most workers believe that filtration reduces the concentration of virus. No one has yet determined to what degree filtration reduces virus concentration. Juice from mosaic-diseased tobacco plants was diluted 1:20 with water and immediately filtered through a

TABLE 3

Effect of Exposure of Tobacco-Mosaic Virus (Diluted 1:20 with Water) to Various Temperatures for Ten Minutes

Temperature in degrees C.	Test No. 1	Test No. 2	Test No. 3	Test No. 4	Test No. 5	Test No. 6	Total	Average
77	272[a]	311	91	200	...	...	874	219
78	206	200	60	293	...	...	759	190
79	146	138	28	109	...	...	421	105
80	132	141	65	92	...	...	430	108
81	92	247	42	131	23	31	566	94
82	169	86	13	93	95	49	505	84
83	429	123	39	120	12	26	749	125
84	351	183	15	217	14	5	785	131
85	126	114	2	36	4	12	294	49
86	91	35	0	64	0	2	192	32
87	2	6	0	21	0	0	29	5
88	0	0	0	0	0	0	0	0
89	0	0	0	0	0	0	0	0
90	0	0	0	0	0	0	0	0
91	0	0	0	0	0	0	0	0
Unheated controls	334	376	222	369	260	199	1760	293

[a] Figures represent numbers of lesions produced upon 5 leaves of *Nicotiana glutinosa* by inoculation with virus sample indicated.

Berkefeld filter candle of the "N" grade. This solution was kept frozen until needed. Four thermal death-point determinations were made with juice that had been treated in this manner. The results of these tests are shown in table 5. In three of the tests, the thermal death point was found to be 84° C., in one test 85° C. The variation is obviously the result of experimental procedure, since the virus samples were identical in each case. This thermal death point is three degrees below that of unfiltered juice diluted 1:20 with water. It is, therefore,

concluded that filtration lowers the thermal death point of tobacco-mosaic virus, although it is realized that freezing also may have altered the resistance of the virus to heat.

Effect of addition of gelatin on thermal death point. In order to obtain more information regarding the influence of concentration of solids

TABLE 4

Effect of Exposure of Tobacco-Mosaic Virus (Diluted 1:20 with Healthy Extract) to Various Temperatures for Ten Minutes

Temperature in degrees C.	Test No. 1	Test No. 2	Test No. 3	Test No. 4	Test No. 5	Total	Average
81	993[a]	1796	194	2100	974	6057	1211
82	378	1675	192	1250	1300	4795	959
83	441	231	98	2303	646	3719	744
84	498	1115	61	456	889	3019	604
85	395	250	70	512	342	1569	314
86	274	1367	20	184	75	1920	384
87	120	397	10	8	0	535	107
88	90	25	3	36	1	155	31
89	4	12	2	11	9	38	8
90	0	2	0	1	0	3	1
91	0	0	0	0	0	0	0
92	0	0	0	0	0	0	0
93	0	0	0	0	0	0	0
Unheated controls	906	1735	270	3240	3320		
	1120	1928	470				
						19932	1533
	2134	1460	162				
	2950		237				
Healthy juice alone	0	0	0	0	0	0	0

[a] Figures represent numbers of lesions produced upon 5 leaves of *Nicotiana glutinosa* by inoculation with virus sample indicated.

on thermal relations of tobacco-mosaic virus, thermal death-point determinations were made with a virus sample to which gelatin had been added. Enough gelatin was added to each solution tested to bring the concentration of gelatin in the sample to two to four per cent by weight. Some slight dilution of the virus sample resulted from this treatment, but the dilution was not significantly large. The re-

TABLE 5

Effect of Exposure of Tobacco-Mosaic Virus (Diluted 1:20 with Water and Filtered through Berkefeld "N" Filter Candle) to Various Temperatures for Ten Minutes

Temperature in degrees C.	Test No. 1	Test No. 2	Test No. 3	Test No. 4	Total	Average
76	...	13[a]	36	14	63	21
77	...	20	27	36	83	28
78	41	21	11	10	83	21
79	20	12	5	19	56	14
80	15	5	11	5	36	9
81	14	9	14	8	45	11
82	7	9	4	7	27	7
83	2	4	3	6	15	4
84	0	0	0	1	1	0.25
85	0	0	0	0	0	0
86	0	0	0	0	0	0
87	0	0	0	0	0	0
Unheated controls	121	72	127	94		
		35	51	144	822	91
		58	120			

[a] Figures represent numbers of lesions produced upon 5 leaves of *Nicotiana glutinosa* by inoculation with virus sample indicated.

TABLE 6

Effect of Exposure of Tobacco-Mosaic Virus (in Undiluted Juice Containing Four Per Cent Gelatin) to Various Temperatures for Ten Minutes

Temperature in degrees C.	Test No 1	Test No. 2	Total	Average
88	2246[a]	360	2606	1303
89	775	60	835	418
90	262	33	295	148
91	42	1	43	22
92	5	0	5	3
93	0	0	0	0
94	0	0	0	0
95	0	0	0	0
Unheated controls	2250	1619	3869	1935

[a] Figures represent numbers of lesions produced upon 5 leaves of *Nicotiana glutinosa* by inoculation with virus sample indicated.

sults of two tests with undiluted juice of mosaic-diseased tobacco plants to which gelatin had been added are given in table 6. In one

TABLE 7

Effect of Exposure of Tobacco-Mosaic Virus (Diluted 1:20 with Water and Containing Four Per Cent Gelatin) to Various Temperatures for Ten Minutes

Temperature in degrees C.	Test No. 1	Test No. 2	Test No. 3	Test No. 4	Total	Average
81	44[a]	132	179	19	374	94
82	21	260	210	40	531	133
83	26	390	124	19	559	140
84	22	52	88	24	186	47
85	7	141	25	8	181	45
86	4	26	11	4	45	11
87	1	9	0	2	12	3
88	0	0	0	0	0	0
89	0	0	0	0	0	0
90	0	0	0	0	0	0
91	0	0	0	0	0	0
Unheated controls	49	519	193	220	981	245

[a] Figures represent numbers of lesions produced upon 5 leaves of *Nicotiana glutinosa* by inoculation with virus sample indicated.

TABLE 8

Effect of Exposure of Tobacco-Mosaic Virus to Different Temperatures for One Minute

Temperature in degrees C.	Test No. 1	Test No. 2	Test No. 3	Test No. 4	Total	Average
92	867[a]	3600	1280	2108	7855	1964
93	338	747	324	1870	3279	820
94	25	251	179	404	859	215
95	0	0	43	3	46	12
96	0	0	0	0	0	0
97	0	0	0	0	0	0
98	0	0	0	0	0	0
Unheated controls	7360	10330	11800	10880	40370	10093

[a] Figures represent numbers of lesions produced upon 16 leaves of Early Golden Cluster beans by inoculation with virus sample indicated.

test with this solution, the thermal death point was found to be 93° C. for ten minutes, in the other test, 92° C. The results of four tests with

juice diluted 1:20 and containing two to four per cent gelatin are given in table 7. In three of these tests, the thermal death point was found to be 88° C., and in one test it was 87° C. for ten minutes. The thermal death points of tobacco-mosaic virus in solutions containing

TABLE 9

Effect of Exposure of Tobacco-Mosaic Virus (Diluted 1:20 with Water) to Various Temperatures for One Minute

Temperature in degrees C.	Test No. 1	Test No. 2	Test No. 3	Test No. 4	Total	Average
87	1536[a]	648	408	479	3071	768
88	701	336	249	136	1422	356
89	528	71	128	128	855	214
90	208	93	92	11	404	101
91	16	5	6	1	28	7
92	0	0	0	0	0	0
93	....	0	0	0	0	0
Unheated controls	8600	4400	3306	3516	19822	4956

[a] Figures represent numbers of lesions produced upon 16 leaves of Early Golden Cluster beans by inoculation with virus sample indicated.

TABLE 10

Effect of Exposure of Tobacco-Mosaic Virus to a Temperature of 90° C.

Time of exposure	Test No. 1	Test No. 2	Test No. 3	Total	Average
20 min.	829[a]	7	179	1015	338
30 "	11	1	110	122	41
40 "	30	0	88	118	39
50 "	1	0	57	58	19
60 "	2	0	3	5	2
70 "	0	0	4	4	1
80 "	0	0	0	0	0
Unheated controls	9330	5688	10800	25818	8606

[a] Figures represent numbers of lesions produced upon 16 leaves of Early Golden Cluster beans by inoculation with virus sample indicated.

two to four per cent gelatin were identical with those in similar solutions that did not contain gelatin. It is concluded that the addition of this quantity of gelatin has no detectable influence upon the thermal death point.

TABLE 11

Effect of Exposure of Tobacco-Mosaic Virus to a Temperature of 85° C.

Time of exposure	Test No. 1	Test No. 2	Test No. 3	Total	Average
4 hrs.	416[a]	1500	243	2159	720
8 "	64	230	631	925	308
12 "	164	185	408	757	252
16 "	48	6	80	134	45
20 "	9	7	8	24	8
24 "	8	5	24	37	12
28 "	4	0	1	5	2
32 "	0	0	0	0	0
36 "	—	0	—	0	0
Unheated controls	6864	7822	5500	20186	6729

[a] Figures represent numbers of lesions produced upon 16 leaves of Early Golden Cluster beans by inoculation with virus sample indicated.

TABLE 12

Effect of Exposure of Tobacco-Mosaic Virus to a Temperature of 80° C.

Time of exposure	Test No. 1	Test No. 2	Test No. 3	Test No. 4	Test No. 5	Total	Average
1 day	1112[a]	6688	1528	3088	3422	15838	3168
2 days	705	1048	1360	2747	5244	11104	2221
3 "	184	1545	805	2096	1728	6358	1272
4 "	133	444	312	680	860	2429	486
5 "	84	374	112	427	.....	997	249
6 "	18	280	35	640	193	1166	233
7 "	19	78	11	201	101	410	82
8 "	8	19	4	99	89	219	44
9 "	1	6	2	17	21	47	9
10 "	0	0	0	6	23	29	6
11 "	.....	.....	3	1	0	4	1
12 "	.....	.....	0	0	0	0	0
13 "	.....	.....	.....	0	0	0	0
14 "	.....	.....	.....	0	.....	0	0
15 "	.....	.....	.....	0	.....	0	0
Unheated controls	13300	11400	8000	10600	9152	52452	10490

[a] Figures represent numbers of lesions produced upon 16 leaves of Early Golden Cluster beans by inoculation with virus sample indicated.

Thermal Death Point: One-Minute Exposure

In all the experiments reported above, a ten-minute interval was taken as a standard in determining the thermal death point. It is of interest to know the thermal death point of tobacco-mosaic virus for a one-minute exposure. In order to determine this, tests were made with undiluted juice of mosaic-diseased tobacco plants and with juice diluted 1:20 with water. Results of these tests are described below.

Undiluted juice. The results of four tests with undiluted juice are given in table 8. In two of these tests, the thermal death point for a one-minute exposure was found to be 95° C.; in the other two tests,

TABLE 13

Effect of Exposure of Tobacco-Mosaic Virus to a Temperature of 75° C.

Time of exposure	Test No. 1	Test No. 2	Test No. 3	Total	Average
10 days	1466[a]	1866	1600	4932	1644
15 "	263	192	33	488	163
20 "	21	19	20	60	20
25 "	17	11	7	35	12
30 "	21	8	5	34	11
35 "	3	6	0	9	3
40 "	0	0	0	0	0
45 "	0	0	0	0	0
Unheated controls	6044	3916	5964	15924	5308

[a] Figures represent numbers of lesions produced upon 16 leaves of Early Golden Cluster beans by inoculation with virus sample indicated.

it was 96° C. Considerable inactivation of virus occurred at 92°C. in all four cases, and there was a gradual diminution in virus concentration with rise in temperature. The results indicate, however, that there is a sharper end point in the case of a one-minute exposure than in the case of a ten-minute exposure.

Juice diluted 1:20 with water. Four tests were made in order to determine the effect of dilution on the thermal death point for a one-minute exposure. As in previous experiments, freshly extracted juice of mosaic-diseased tobacco plants diluted 1:20 with water was used. Results of these four tests are presented in table 9. In all four tests, the thermal death point for a one-minute exposure was found to be

TABLE 14

Effect of Exposure of Tobacco-Mosaic Virus to a Temperature of 68° C.

Time of exposure	Test No. 1	Test No. 2	Test No. 3	Test No. 4	Test No. 5	Total	Average
4 hrs.	11260	7377	....	....	.....	18637	9319
12 "	5250	3700	....	....	.....	8950	4475
24 "	7000	4600	....	....	.....	11600	5800
2 days	5000	3275	....	....	.....	8275	4138
3 "	5350	3303	....	....	.....	8653	4327
5 "	2369	811	....	....	.....	3180	1590
8 "	4450	1334	....	....	.....	5784	2892
10 "	2525	1045	....	....	.....	3570	1785
14 "	1635	655	....	....	.....	2290	1145
18 "	.....	.....	....	....	189	189	189
19 "	905	421	....	....	.....	1326	663
20 "	.....	.....	....	....	91	91	91
31 "	.....	.....	84	85	.....	169	85
33 "	.....	.....	185	....	.....	185	185
37 "	.....	.....	....	....	12	12	12
39 "	.....	.....	52	....	8	60	30
40 "	.....	.....	45	....	.....	45	45
42 "	.....	.....	24	....	.....	24	24
44 "	.....	.....	....	....	0	0	0
46 "	.....	.....	....	....	2	2	2
48 "	.....	.....	....	5	.....	5	5
50 "	.....	.....	....	5	0	5	3
52 "	.....	.....	0	2	1	3	1
53 "	5	0	....	....	.....	5	3
54 "	.....	.....	0	1	0	1	1
56 "	.....	.....	0	37	.....	37	19
58 "	.....	.....	0	4	.....	4	2
60 "	.....	.....	....	26	.....	26	26
62 "	.....	.....	....	9	.....	9	9
64 "	.....	.....	....	5	.....	5	5
66 "	.....	.....	....	4	.....	4	4
68 "	.....	.....	....	11	1	12	6
70 "	0	0	....	4	1	5	1
Unheated controls	17400	17800	....	....	.....	.....	.....
	9100	7400	5760	8280	13300	98440	10938
	10600	8800	....	....	.....	.....	.....

92° C. Partial inactivation occurred at 87° C., the lowest temperature tested.

TABLE 15

Effect of Exposure of Tobacco-Mosaic Virus (Diluted 1:20 with Water) to a Temperature of 85° C.

Time of exposure	Test No. 1	Test No. 2	Test No. 3	Test No. 4	Total	Average
20 mins.	....	3[a]	5	114	122	41
30 "	2	0	0	18	20	5
40 "	1	0	2	12	15	4
50 "	1	1	5	10	17	4
60 "	0	0	0	1	1	1
70 "	0	0	0	0	0	0
80 "	0	....	0	0	0	0
Unheated controls	8600	4155	4225	3200	20180	5045

[a] Figures represent numbers of lesions produced upon 16 leaves of Early Golden Cluster beans by inoculation with virus sample indicated.

TABLE 16

Effect of Exposure of Tobacco-Mosaic Virus (Diluted 1:20 with Water) to a Temperature of 80° C.

Time of exposure	Test No. 1	Test No. 2	Test No. 3	Test No. 4	Total	Average
1 hr.	2014[a]	430	399	146	2989	747
2 hrs.	493	206	147	200	1046	261
3 "	132	171	291	141	735	184
4 "	76	58	107	45	286	72
5 "	91	93	194	11	389	97
6 "	6	12	220	9	247	62
7 "	5	94	123	2	224	56
8 "	1	12	48	1	62	16
9 "	0	7	45	0	52	13
10 "	5	9	9	0	23	6
11 "	0	0	2	4	6	2
12 "	0	0	1	0	1	0.25
13 "	0	0	0	0	0	0
14 "	0	0	0	0	0	0
15 "	0	1	0	0	1	0.25
16 "	0	0	0	0	0	0
Unheated controls	2014	4186	4784	5500	26990	3856
	4600	2122	3784			

[a] Figures represent numbers of lesions produced upon 16 leaves of Early Golden Cluster beans by inoculation with virus sample indicated.

TABLE 17

Effect of Exposure of Tobacco-Mosaic Virus (Diluted 1:20 with Water) to a Temperature of 75° C.

Time of exposure	Test No. 1	Test No. 2	Test No. 3	Test No. 4	Total	Average
12 hrs.	990[a]	100	512	233	1835	459
24 "	355	4	184	40	583	146
36 "	4	0	20	1	25	6
48 "	0	3	68	0	71	18
60 "	0	0	2	0	2	1
72 "	0	0	0	0	0	0
84 "	....	....	0	0	0	0
Unheated controls	2448	2915	1228	1511	8102	2026

[a] Figures represent numbers of lesions produced upon 16 leaves of Early Golden Cluster beans by inoculation with virus sample indicated.

TABLE 18

Effect of Exposure of Tobacco-Mosaic Virus (Diluted 1:20 with Water) to a Temperature of 68° C.

Time of exposure	Test No. 1	Test No. 2	Test No. 3	Total	Average
2 days	....	906[a]	1070	1976	988
3 "	455	656	456	1567	522
4 "	235	354	576	1165	388
5 "	114	....	....	114	114
6 "	171	293	426	890	297
7 "	624	49	62	735	245
8 "	144	61	135	340	113
9 "	37	92	322	451	150
10 "	3	107	19	129	43
11 "	2	2	624	628	209
12 "	167	11	260	438	146
13 "	22	67	394	483	161
14 "	0	0	7	7	2
15 "	5	9	11	25	8
16 "	0	0	0	0	0
17 "	0	5	17	22	7
18 "	0	0	13	13	4
19 "	0	1	8	9	3
20 "	0	0	0	0	0
Unheated controls	3912	760	356	5028	1676

[a] Figures represent numbers of lesions produced upon 16 leaves of Early Golden Cluster beans by inoculation with virus sample indicated.

Thermal Death Time for Various Temperatures

Undiluted juice. The thermal death time for virus in freshly extracted juice of mosaic-diseased tobacco was determined for temperatures of 90°, 85°, 80°, 75°, and 68° C. In all cases the virus concentration remaining after heating was measured by inoculation of leaves of Early Golden Cluster beans. Results of tests at these temperatures are shown in tables 10 to 14. Table 10 shows the results of three tests with virus samples held at 90° C.; table 11, the results of three tests at 85° C.; table 12, the results of five tests at 80° C.; table 13, the results of three tests at 75° C.; and table 14, the results of five tests at 68° C. It is concluded from these tests that tobacco-mosaic virus in undiluted juice is completely inactivated when held at 90° C. for 80 minutes, at 85° C. for 32 hours, at 80° C. for 12 days, or at 75° C. for 40 days. It was not completely inactivated when held at a temperature of 68° C. for as long as 70 days.

Diluted juice. Tests were made to determine the thermal death time at various temperatures for virus in juice of mosaic-diseased tobacco diluted 1:20 with water. Tables 15 to 18 show the number of lesions produced on Early Golden Cluster beans by inoculation with virus heated to these temperatures. Table 15 shows results of four tests with diluted juice held at 85° C.; table 16, results of four tests at 80° C.; table 17, results of four tests at 75° C.; and table 18, results of three tests at 68° C. These results show that virus in freshly extracted juice, diluted 1:20 with water, is completely inactivated when heated to 85° C. for 70 minutes, to 80° C. for 13 hours, to 75° C. for 72 hours, or to 68° C. for 20 days.

Curves Showing Rate of Inactivation

Some of the data obtained in this investigation are plotted in the form of curves and presented in figures 1 and 2. Figure 1 shows the rate of inactivation of tobacco-mosaic virus in undiluted juice held at a temperature of 68° C. Data from which this curve was drawn were taken from table 14. The curve shows clearly the gradual diminution in virus concentration due to heating.

The curves in figure 2 were drawn with time plotted on a logarithmic scale and temperature on an arithmetic scale. They show the time-temperature relation for complete inactivation of virus. The points

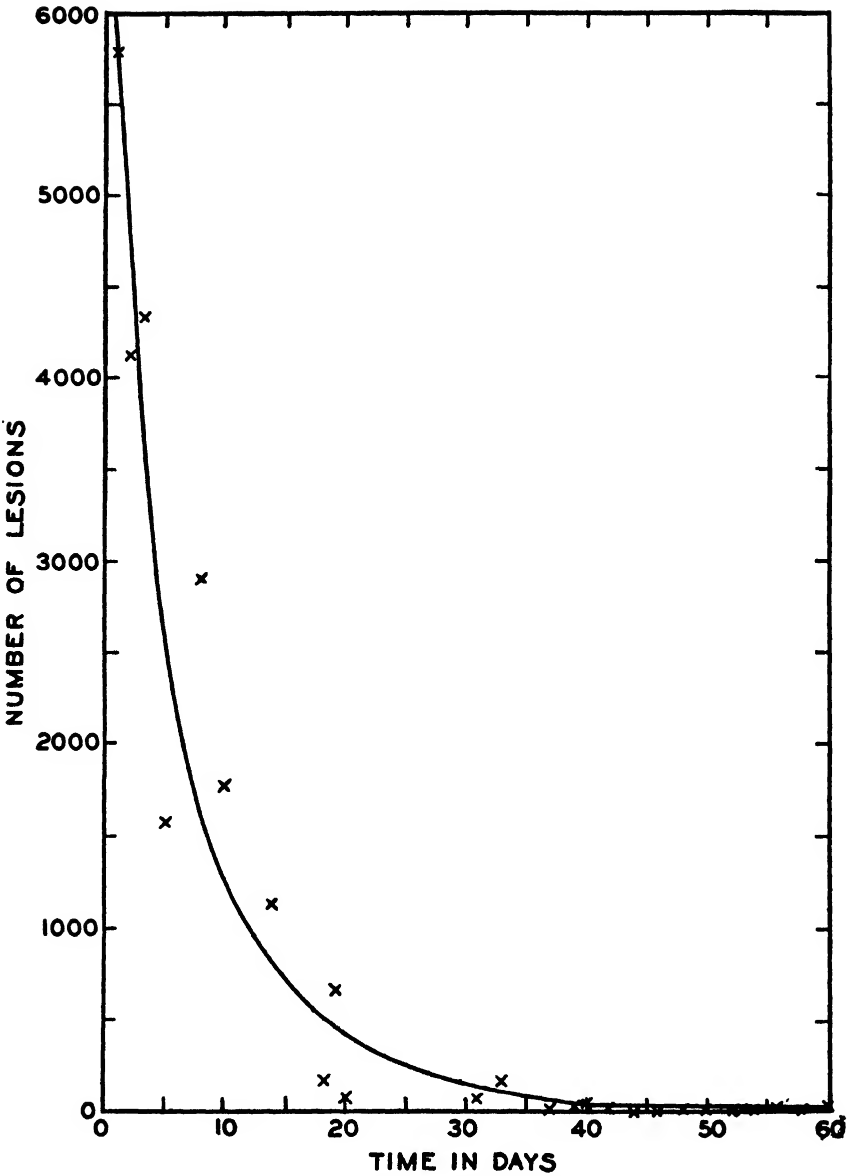

Fig. 1. Curve showing the rate of inactivation of tobacco-mosaic virus (in freshly expressed juice of diseased tobacco plants) when held at a temperature of 68°C. The points on the curve show the average number of lesions produced on leaves of Early Golden Cluster beans by inoculation with virus samples heated for the length of time indicated.

on the curves represent the times, shown in tables 1 to 3 and 8 to 18, for complete inactivation of virus at the temperatures indicated. With these curves, it is possible to estimate the thermal death time for any temperature between 68° C. and 96° C., or to calculate the thermal death temperature for any period between one minute and 40 days. Although the curve for inactivation of virus in undiluted juice and that for virus in juice diluted 1:20 are similar in shape, they are

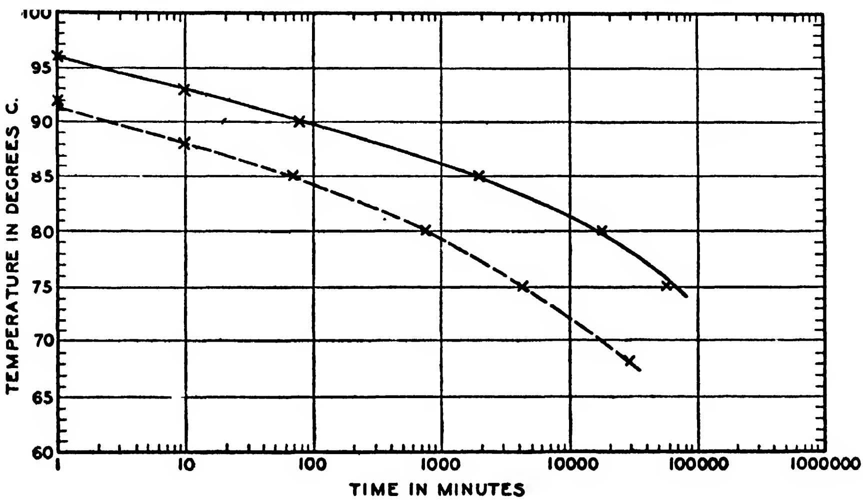

FIG. 2. Thermal death-time curves of tobacco-mosaic virus. The solid line shows the time required for complete inactivation of virus in freshly expressed juice of mosaic-diseased plants at the temperatures indicated; the broken line, the time required for inactivation of virus in juice diluted 1:20 with water.

closer together at the higher temperatures tested than at the lower. This appears to show that dilution alters the thermal death time more at low temperatures than at high temperatures.

DISCUSSION

The results obtained in this investigation are in fairly close agreement with those of other workers. The seemingly higher thermal death point obtained by the writer and also by Johnson and Grant (8) may be due to the use of the rubbing method of inoculation, which allows detection of minute quantities of virus. It has been shown that the thermal death point is influenced by concentration of virus and by

concentration of solids in the dispersing medium. It may be that such factors would be operative in the case of virus samples obtained from host plants grown under different environmental conditions. Differences between results observed by various investigators, as well as differences between certain samples of virus used by the writer, may possibly be due to differences in plants from which the virus samples were obtained. It is of importance that such factors as concentration of solids and nature of the dispersing medium be taken into consideration when comparing the thermal death points of different viruses or even of the same virus from different sources.

Another factor that may account for some of the variation obtained in different tests with tobacco-mosaic virus is the difficulty of maintaining a uniform temperature over a considerable period of time. Examination of the curves presented in figure 2 shows that a slight change in temperature causes a significantly large change in thermal death time of the virus. Variation in temperature of as much as one degree C. might result in a considerably shorter or considerably longer thermal death time.

Thermal death-time curves for several thermophilic organisms were published by Bigelow (3). These curves, on semilogarithmic paper, are straight lines except at temperatures below 105° C., where they tend to turn downward. It is of interest that the thermal death-time curves of tobacco-mosaic virus (Fig. 2) resemble those shown by Bigelow for thermophilic organisms. These curves for tobacco-mosaic virus are almost straight lines at the high temperatures but bend downward sharply at temperatures below 80° and 85° C.

The virus of tobacco mosaic is more resistant to heat than most of the other plant viruses that have been studied up to the present time. No other plant virus is known to have a higher thermal death point. That it is far more resistant than are most vegetative bacteria is shown by its ability to withstand the temperature of pasteurization for many days.

SUMMARY

The local-lesion method for measuring virus concentration was used in a quantitative study of the rate of inactivation of tobacco-mosaic virus by heat. It was found that virus in undiluted juice of mosaic-

diseased tobacco plants was inactivated in one minute at 96° C., in ten minutes at 93° C., in 80 minutes at 90° C., in 32 hours at 85° C., in 12 days at 80° C., and in 40 days at 75° C. It was not completely inactivated when held for 70 days at 68° C. Virus in juice of mosaic-diseased tobacco plants diluted 1:20 with water was completely inactivated in one minute at 92° C., in ten minutes at 88° C., in 70 minutes at 85° C., in 13 hours at 80° C., in 72 hours at 75° C., and in 20 days at 68° C.

Tables giving measurements of virus concentration after heating for different periods of time at the same temperature and for different temperatures for the same length of time are presented in the text. These measurements indicate that inactivation of tobacco-mosaic virus by heat is a gradual process that goes on more rapidly at first than after partial inactivation has occurred.

Time-temperature curves for inactivation of mosaic virus in undiluted juice and in juice diluted 1:20 with water were drawn on semilogarithmic paper and are presented in the text. They show the time required for complete inactivation of virus at temperatures between 68° and 96° C.

Thermal death-point determinations made with virus in juice diluted with water and in juice diluted with extract of healthy tobacco indicate that inactivation of virus is influenced by concentration of virus and concentration of solids in the dispersing medium.

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ISOLATION OF YELLOW-MOSAIC VIRUSES FROM PLANTS INFECTED WITH TOBACCO MOSAIC

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INTRODUCTION

The mosaic disease of tobacco, as is well known, is characterized by a blotching of the leaves with light and dark green areas. These areas produce the mottling or mosaic pattern, from which the disease derives its name. In addition to this mottling, small bright yellow spots occasionally occur on the leaves. They are few in number and may be easily overlooked. They are found, however, on most tobacco plants at some time in the course of systemic infection.

McKinney (4, 5, 6) has pointed out that the bright yellow areas contain a virus that differs from that of ordinary tobacco mosaic. He was able to isolate a yellow mosaic by cutting out the small yellow spots and inoculating from them. McKinney stated that the association of ordinary tobacco-mosaic virus and the yellow-mosaic virus might not constitute a contamination in the usual sense. He suggested that the yellow-mosaic virus might have arisen as the result of a mutation, although a satisfactory interpretation could not be given at that time because so few data were available. Dufrénoy (1) found that juice from bright yellow areas produced local yellow lesions when inoculated into mature leaves of tobacco.

Experiments were undertaken to determine whether the virus contained in the bright yellow spots is present in the original inoculum, occurs as the result of contamination, or arises in tissues invaded by the disease. The purpose of this paper is to describe the experiments and to report the results obtained.

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Materials and Methods

The tobacco-mosaic virus used in these experiments was of the ordinary field type and produced typical tobacco-mosaic symptoms.

The plants used in studies on bright yellow spots were *Nicotiana tabacum* L. variety Turkish, *N. sylvestris* Spegaz. & Comes, and *Lycopersicon esculentum* Mill. *N. glutinosa* L. was used in attempts to obtain samples of tobacco-mosaic virus free from all other viruses. *N. langsdorffii* Schrank was used in attempts to isolate pure samples of yellow-mosaic viruses.

All plants were grown in soil in 4-inch or 6-inch porous clay pots or in shallow wooden flats. Special precautions were taken to prevent accidental infection of inoculated plants with other viruses. Touching the plants during watering and other greenhouse operations was carefully avoided. The greenhouses were fumigated frequently to control insects.

The following inoculation methods were used:

1. *Pot-label method.* Two small sterile wooden pot labels were used to break off and macerate a piece of diseased leaf. Transfers of virus to other plants were made by rubbing one or two small leaves with these pot labels. The advantage of this method is that it permits the easy transfer of several different viruses without contaminating the hands.

2. *Single pin-puncture method.* A sterilized #00 insect pin was inserted into that portion of a diseased leaf from which a transfer was to be made, and then into a small leaf (0.5 to 1.5 cm. long) of a healthy young tobacco plant. This method was used in transferring virus from bright yellow spots.

3. *Glass-spatula method.* A disk of diseased leaf tissue was cut out by means of a sterile cork borer and macerated on a glass slide with a glass spatula similar to that described by Samuel (7). Inoculations were made by rubbing leaves with the glass spatula. This method was used in making transfers from local necrotic lesions occurring on leaves of *Nicotiana glutinosa* and *N. langsdorffii.*

Experimental Procedure

In preliminary experiments it was found that bright yellow spots from which a yellow-mosaic virus could be isolated occurred on leaves

of *Nicotiana tabacum* variety Turkish, *N. sylvestris*, and *Lycopersicon esculentum*, whenever such plants were inoculated with tobacco-mosaic virus. The yellow-mosaic virus obtained from these yellow spots produced symptoms that differed from those of tobacco mosaic. Plants inoculated with mixtures of the virus of tobacco mosaic and that of the yellow mosaic developed symptoms that were different from those produced by either virus alone. When tobacco plants were inoculated with mixtures containing various proportions (1:1 to 1:100) of the 2 viruses, they were easily distinguished from those infected with only one virus.

It was found that the yellow-mosaic virus, rubbed over leaves of *Nicotiana glutinosa*, produced local necrotic lesions that appeared identical with those produced on this plant by tobacco-mosaic virus. When solutions containing mixtures of these 2 viruses were rubbed over leaves of *N. glutinosa*, there appeared necrotic lesions all of which looked alike. It was found, however, that when single necrotic lesions from such leaves were used to inoculate young tobacco plants, a high percentage of the plants produced symptoms characteristic of infection with one virus only. These results suggest that single necrotic lesions offer a means of separating the virus of tobacco mosaic from that of the yellow mosaic. The method was used in attempts to obtain samples of tobacco-mosaic virus that would be free from the virus of yellow mosaic. These attempts were carried out in the following manner. Dilute solutions of the expressed juice of tobacco plants infected with tobacco mosaic were rubbed over leaves of *Nicotiana glutinosa* plants. A number of necrotic lesions were produced. Virus from isolated single lesions was transferred through a series of *N. glutinosa* plants, each being inoculated with virus from a single lesion of the preceding plant. Necrotic lesions on these plants were frequently tested for the presence of yellow-mosaic virus by inoculating young tobacco plants from single lesions. These plants always produced typical tobacco-mosaic symptoms. No bright yellow primary lesions, such as would have indicated that the virus of yellow mosaic was present, ever appeared. It is believed, therefore, that the virus of tobacco mosaic obtained from necrotic lesions was free from that of yellow mosaic.

With tobacco-mosaic virus obtained by this method, an experiment

was next undertaken to determine whether yellow spots would occur on leaves of plants systemically infected and protected against contamination in the greenhouse.

Plants used in the experiment were tobacco, *Nicotiana sylvestris*, and tomato. Twenty healthy young plants of each species, which were later to be inoculated, were placed in the central portion of a greenhouse bench. Twenty similar plants, which were later to serve as controls, were placed so as to completely surround the test plants. This arrangement left the controls in the location where they would be most exposed to accidental infection, if any should occur.

All plants were kept under close observation for at least a week before being inoculated. Neither symptoms of tobacco mosaic nor chlorotic spots of any kind appeared on the plants during this preliminary observation period.

Inoculations on the twenty plants in the center of the group were made with virus from single necrotic lesions on *Nicotiana glutinosa* leaves. The tobacco and tomato plants were inoculated from lesions obtained on the 10th serial transfer, whereas plants of *N. sylvestris* were inoculated from lesions obtained on the 15th serial transfer. The glass-spatula method was used in making the inoculations. Of the 20 plants of each species inoculated, infection was obtained in 16 tobacco, 20 *N. sylvestris*, and 10 tomato plants. All of the 60 control plants remained healthy during the course of the experiments.

Within 10 days to 2 weeks after inoculation, some of the mottled leaves of the infected plants showed small bright yellow spots, similar to those observed in previous experiments. Other yellow spots continued to appear as long as the plants were held. Typical bright yellow spots are shown in figure 1. Of the 46 plants infected, all except 2 (1 of *Nicotiana sylvestris* and 1 of tomato) finally produced yellow spots. A total of 157 spots appeared during the period of 1 month following inoculation. Table 1 gives a summary of the results obtained. Since no chlorotic spots appeared on the 60 control plants in this experiment, it is concluded that the yellow spots on infected plants were not the result of contamination or of accidental infection.

During the course of the studies, attempts were made to isolate virus from a number of the yellow spots. Leaves bearing yellow spots were removed from the plants by means of sterile scalpels. The single

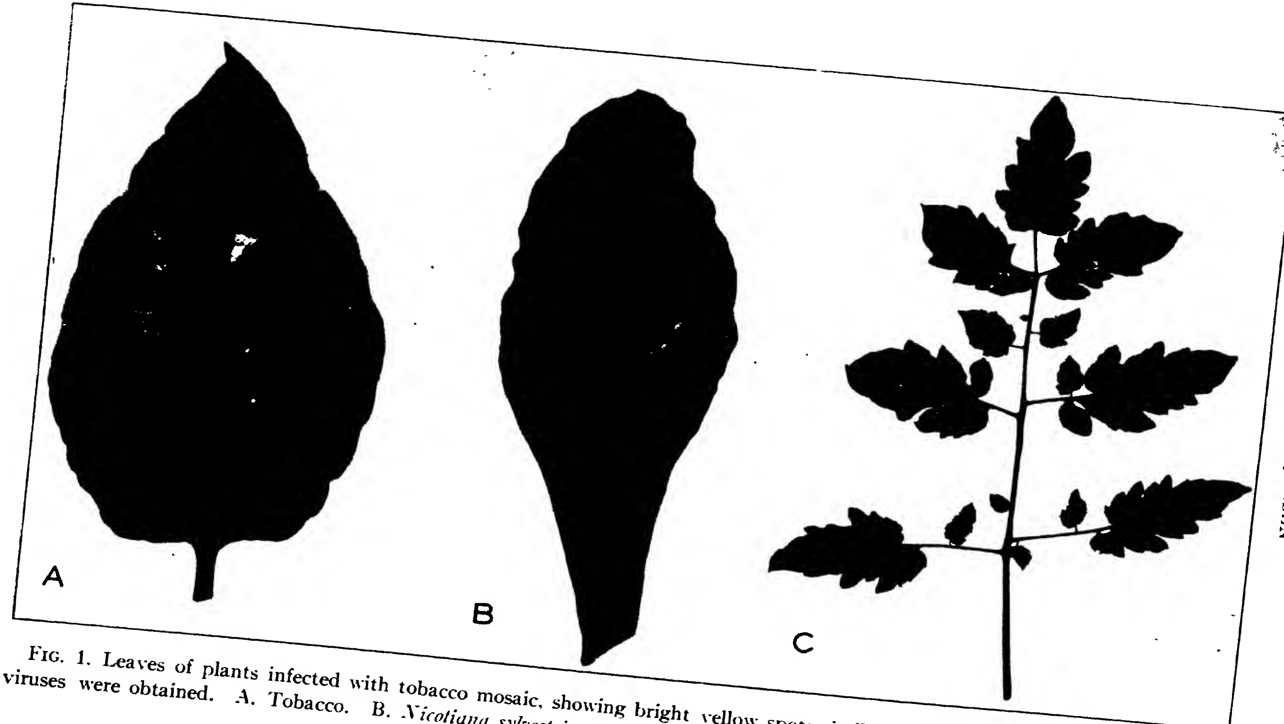

FIG. 1. Leaves of plants infected with tobacco mosaic, showing bright yellow spots similar to those from which yellow-mosaic viruses were obtained. A. Tobacco. B. *Nicotiana sylvestris.* C. Tomato.

pin-puncture method was used in attempts to transfer virus from the yellow spots to young tobacco plants. Three to 5 days after inoculation, some of the small plants usually showed symptoms of infection. Six to 7 days after inoculation, the virus from each small diseased plant was transferred by the pot-label method to several healthy young Turkish tobacco plants. The symptoms on these plants were carefully observed. Plants showing infection with tobacco mosaic or with a mixture of tobacco mosaic and yellow mosaic were discarded. Those showing only symptoms of yellow mosaic were held separately. Twenty-six isolations of yellow-mosaic virus were made from yellow spots.

TABLE 1

Summarized Results of Attempts to Isolate Virus from Yellow Spots in the Leaves of Nicotiana spp. and Tomato

	N. tabacum	*N. sylvestris*	*L. esculentum*
Plants inoculated with tobacco-mosaic virus from necrotic lesions	20	20	20
Number of plants infected	16	20	10
Plants with yellow spots on leaves	16	19	9
Total number of yellow spots on infected plants	68	54	35
Isolations attempted	36	36	25
Yellow-mosaic viruses obtained	12	8	6

When the symptoms produced by inoculating plants from the yellow spots were studied, it became apparent that a number of different yellow-mosaic viruses had been obtained. It was observed that, while all plants inoculated with virus obtained from different yellow spots produced more or less similar primary yellow lesions, some produced distinctly different systemic symptoms. These observations suggested that the different yellow spots contained different viruses. Since these viruses were not the result of contamination or accidental infection and, since their presence in the samples of tobacco-mosaic virus used for inoculation could not be demonstrated, even though repeated tests were made, it appears that they must have arisen in the infected plants.

For the purpose of comparing the viruses obtained from different

yellow spots, attempts were made to inoculate various species of plants. Among the plants tested were the following: *Nicotiana glutinosa* L.; *N. glauca* R. Grah.; *N. rustica* L.; *N. sylvestris* Spegaz. & Comes; *N. paniculata* L.; *N. langsdorffii* Schrank; *N. acuminata* Hook.; *Lycopersicon esculentum* Mill.; *Physalis angulata* L.; *Solanum melongena* L. var. Hangchow Long.

Observations on the various kinds of plants inoculated with yellow-mosaic viruses showed that a number of the virus samples obtained from different yellow spots produced different symptoms on these plants as well as on tobacco.

On the basis of production of systemic symptoms in tobacco, the viruses appear to fall into 3 groups. Those in the first group produced general systemic infection with symptoms resembling tobacco mosaic but characterized by more intense yellowing. The viruses of this group all produced the clearing-of-veins symptom. This symptom was followed by the emergence of several leaves showing mottling and distortion. Such leaves were followed by 3 or 4 healthy-appearing, intensely green leaves. In many instances, these leaves showed no symptoms of disease until they were almost fully expanded, when blotch-like or ring-like yellow areas appeared. These yellow areas usually enlarged slowly until large portions of the leaves were involved. Later stages of the disease were characterized by the production of leaves that were more or less yellowed. The intensity of yellowing of the diseased leaf tissue varied with the different viruses of the group. Some viruses produced bright yellow chlorotic areas, others produced greenish yellow areas. The viruses producing the more intense yellow symptoms often affected the leaf tissue so severely that complete collapse and necrosis occurred. This was especially true of leaves involved in the clearing-of-veins symptom. Except for greater intensity of foliage symptoms the diseases in this group corresponded very closely with tobacco mosaic.

A second group of viruses produced systemic infections, which ordinarily advanced slowly and usually produced no typical clearing-of-veins symptoms. The first symptoms of systemic infection by viruses of this group consisted in the appearance of numerous small yellow spots on 1 or 2 of the young leaves. In some instances, these small spots tended to join together, forming a rough clearing-of-

veins pattern; in other cases the yellow areas enlarged slowly until most of the leaf was yellowed. Symptoms appeared on leaves higher up on the stem in the form of blotch-like or ring-like areas. Such spots, whether on the veins or elsewhere, enlarged slowly until most or all of the leaf was involved. The later stages of the disease were characterized by a slow enlargement of the existing yellow areas and the appearance of similar yellow areas on some of the younger leaves.

A third group contained a number of viruses that produced few or no symptoms other than the primary yellow lesions on the inoculated leaf. Some of them produced isolated yellow spots on 1 or 2 leaves above the inoculated leaf. Others produced chlorotic oak-leaf patterns, which appeared first on the leaves at the base of the stem and, later, on some of the leaves above. Several different virus samples were observed to produce no symptoms other than those on the inoculated leaf during a period of about a month following inoculation. The yellow lesions at the point of inoculation usually enlarged slowly until the entire leaf became yellow and finally collapsed. Several samples of the viruses produced yellow primary lesions that soon became necrotic.

The viruses of the 3 groups just described differ in the ease with which they are transmitted to healthy plants. Those of the first group are transferred readily, whereas those of the other 2 groups are transferred with considerable difficulty. Some of them are so difficult to transmit mechanically that even severe rubbing of the leaves with undiluted virus extracts does not give a high percentage of infection.

The yellow-mosaic viruses that have been isolated have not yet been sufficiently studied to justify a detailed description of symptoms. Brief descriptions of the most characteristic symptoms of 3 of them in tobacco and a few other plants are given below.

1. *Yellow mosaic, isolation No. 102.* This virus, which belongs in the first group, produces large yellow primary lesions on inoculated leaves of tobacco plants. These are followed by a sharply defined yellow vein-clearing and later by systemically infected leaves that are almost entirely yellow. A leaf of this type is shown in figure 2, A. Systemic symptoms of yellow mosaic are also caused by this virus on tomato, *Nicotiana sylvestris*, *N. glauca*, *N. paniculata*, and *S. melongena* var. Hangchow Long. Local necrotic lesions are produced on *N.*

glutinosa, *N. acuminata*, and *N. langsdorffii*. Systemic necrosis is produced in *N. rustica* and *Physalis angulata*. The symptoms of the disease caused by this virus appear to be similar to those of Tobacco Mosaic No. 6 of Johnson (3), White Mosaic (2), and Aucuba Mosaic (8).

2. *Yellow mosaic, isolation No. 3.* This virus belongs in the third group. It produces large yellow primary lesions of indefinite outline when inoculated into tobacco leaves. These lesions continue to enlarge slowly, extending along the principal veins, as shown in figure 2, B. Usually, no symptoms other than the slowly enlarging spots appear on the plants. The spots finally extend over most of the affected leaf until, after several weeks, the entire leaf collapses. Large yellow spots, few in number, appear occasionally on 1 or 2 leaves above the inoculated leaf, but no vein-clearing or other symptoms of general systemic infection are to be observed. In one experiment, in which 15 Turkish tobacco plants infected with this virus were held about a month, symptoms appeared on leaves other than those inoculated in only 2 plants. This virus produces yellow blotch-like primary lesions on *Nicotiana sylvestris*, tobacco, and tomato; local necrotic lesions on *N. glutinosa* and *N. acuminata*. The virus was transferred to healthy plants with difficulty. So far as is known, the symptoms of the disease caused by this virus on tobacco have not been previously described.

3. *Yellow mosaic, isolation No. 107.* Primary lesions appear on tobacco leaves 4 to 5 days after inoculation with this virus, which also belongs in the third group. These lesions are of indefinite outline, grayish yellow, and soon break down to form blotch-like grayish brown necrotic areas (Fig. 2, C). Several weeks after inoculation, small brown necrotic spots and faint, slightly chlorotic, oak-leaf patterns appear on older leaves near the base of the inoculated plants. This virus produces local necrotic lesions on *Nicotiana sylvestris*, *N. glutinosa*, and *N. acuminata*. It is difficult to transfer it to healthy plants. So far as is known, the disease resulting from infection by this virus has not been previously described. The difference between it and the viruses producing yellow spots is regularly shown when inoculations are made into similar plants in parallel experiments. It is possible that the virus would not produce necrosis on tobacco under

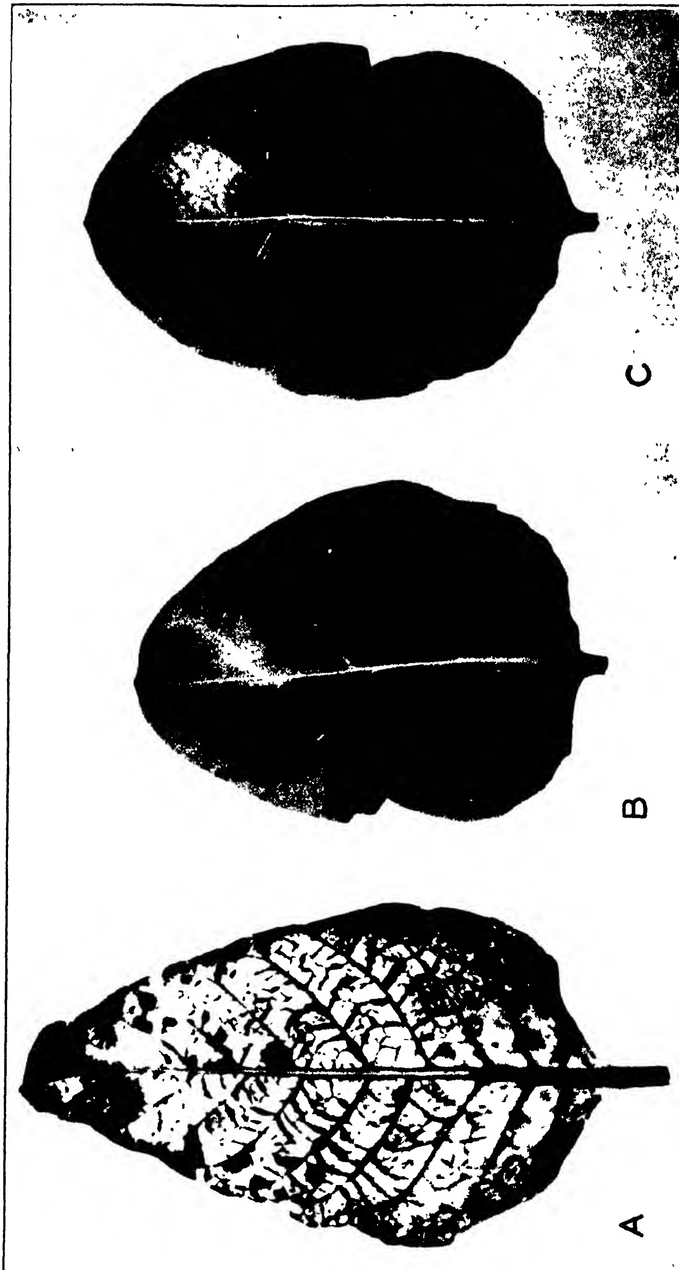

FIG. 2. Leaves of tobacco plants showing symptoms of yellow-mosaic viruses. A. Yellow mosaic, isolation No. 102, mottled leaf. B. Yellow spot, isolation No. 3, primary lesions. C. Necrotic spot, isolation No. 107, primary lesion.

all environmental conditions. In experiments conducted during the spring and summer months under varying light and temperature conditions, it invariably produced the symptoms described.

From these brief descriptions it is seen that the 3 viruses produce distinctive symptoms. Some of the other yellow-mosaic viruses produce less striking differences.

To test the possibility that the small differences might be due to mixtures, many of the viruses were reisolated from single necrotic lesions on leaves of *Nicotiana langsdorffii* in the following way. Each yellow mosaic was transferred to a separate set of *N. langsdorffii* plants. Six or 7 days after inoculation, 10 single necrotic lesions were cut out of the leaves of each set and used to inoculate 10 tobacco plants, each lesion being used on a different plant. In every instance, the 10 inoculated tobacco plants of a set gave identical symptoms. It was, therefore, concluded that the viruses thus tested were free from other viruses and that the differences observed resulted from differences inherent in each of them.

DISCUSSION

Evidence obtained during the course of this investigation indicates that yellow-mosaic viruses arise in bright yellow spots that regularly appear on the leaves of tobacco and tomato plants affected with tobacco mosaic. Some of these viruses cause diseases that differ markedly from each other and remain distinct in serial transfers. Others produce symptoms that differ so slightly that they are almost indistinguishable on the plants to which they have been transmitted. Still others cause diseases that seem identical.

It is believed that the possibility that these viruses may be carried along with the virus of tobacco mosaic in transfers from plant to plant or that they may be accidentally transmitted from some other plant to the tissues in which they appear, has been eliminated. Some of them are difficult to transmit to tobacco. Considering this characteristic, it seems improbable that they would be carried along in ordinary tobacco-mosaic inoculation. Since it is very difficult to recover them from the necrotic lesions they produce on *Nicotiana glutinosa*, it is even more improbable that they would be retained through a series of single necrotic-lesion transfers of tobacco mosaic such as was made in

efforts to obtain pure samples of this virus. No bright yellow spots have ever been observed on uninoculated check plants. They occur only on plants that have tobacco mosaic and are far too numerous to permit their being considered as due to accidental contaminations. It is, therefore, believed that the yellow-mosaic viruses arise from the tobacco-mosaic virus. Although these viruses and the diseases they produce have not yet been sufficiently studied to justify conclusions regarding the extent to which they differ from each other or from the tobacco-mosaic virus, they are considered to be strains of the latter. All of them are readily distinguished from the latter by the symptoms produced in tobacco. Some also differ in showing a lower infectivity and a slower rate of spread from primary lesions. Insofar as has been determined, they have the same host range as tobacco mosaic. In several different plants they produce necrotic primary lesions that are indistinguishable from those of the latter disease. Nothing is known as to the manner in which they arise, or the conditions favoring their production.

Some of the yellow mosaics of Solanaceous plants prevalent in commercial fields and greenhouses may have originated in yellow spots on leaves of plants having tobacco mosaic. It is possible that sucking insects that accidentally feed on the bright yellow spots on such leaves and later on healthy tobacco or tomato plants may thereby isolate yellow-mosaic viruses and start epidemics of such diseases as Tobacco Mosaic No. 6 of J. Johnson (3), the White Mosaic of E. M. Johnson (2), or Aucuba Mosaic (8). Some of the yellow mosaics isolated differ from any that have previously been described.

SUMMARY

Small, bright yellow spots, containing the viruses of yellow mosaics, occasionally appear on leaves of *Nicotiana tabacum* variety Turkish, *N. sylvestris*, and *Lycopersicon esculentum* infected with tobacco mosaic.

Similar yellow spots appear when plants of these species, protected against accidental infection, are inoculated with tobacco-mosaic virus shown to be free from all other viruses.

Twenty-six isolations of yellow-mosaic virus were made from yellow spots. Many of them differ from each other. The symptoms produced by 3 that showed marked differences are briefly described.

Evidence obtained in this investigation indicates that viruses of yellow mosaics arise during multiplication of tobacco-mosaic virus in infected plants.

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